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Ultra-sensitive *in situ* visualization of active glucocerebrosidase molecules

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Supplementary results

Synthesis of the probes

KY170 **4**, and fluorescent probes MDW933 **5** and MDW941 **6** were synthesized as follows. First, core carbocycle **10** was synthesized in 7 steps from D-xylose as described (1). Selective tosylation of the primary alcohol in **1** by treatment with *p*-toluenesulfonyl chloride in CH_2Cl_2 followed by substitution of the tosylate with sodium azide afforded azido alcohol **11** in 72%. The benzyl groups in **11** were removed under the agency of BCl_3 prior to epoxidation. The resulting free hydroxyls were protected as the corresponding benzoyl protective groups using benzoyl chloride in pyridine. Epoxidation of cyclohexene **12** using *in situ* formed methyl(trifluoromethyl)dioxirane gave epoxides **13** and **14** as a separable mixture. Deprotection with sodium methoxide in methanol gave 8-deoxy-8-azidocyclophellitol (KY170, **4**). BODIPY Green-alkyne **15** (green fluorescent) or BODIPY Red-alkyne **16** (red fluorescent) was conjugated to KY170 **4** using copper–catalyzed click chemistry, giving MDW933 **5** and MDW941 **6** respectively. Non-reactive control probes MDW1064 **8** and MDW1065 **9** were synthesized by removing the protective groups in azido alcohol **11** with BCl_3 and subsequent conjugation to either BODIPY Green **15** or BODIPY Red **16**.

Supplementary Scheme 1. Synthesis of KY170 **4**, MDW933 **5** and MDW941 **6**.

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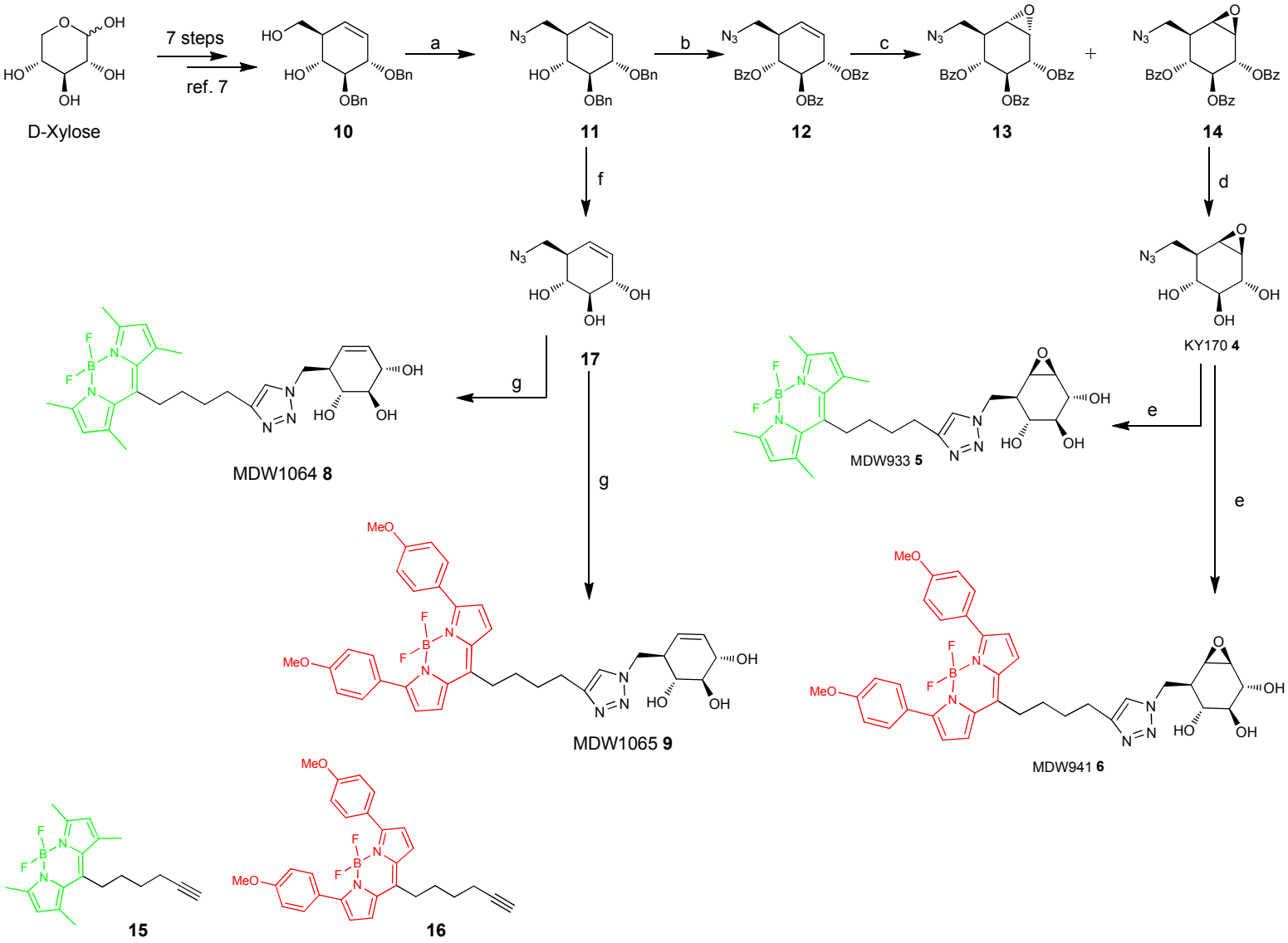

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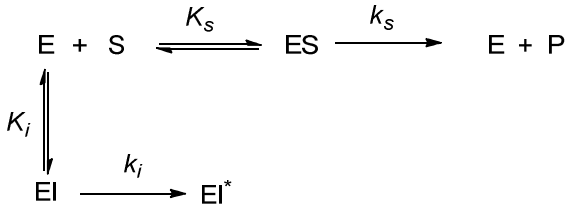
Reagents and conditions: (a) i) *p*-TosCl, Et_3N, CH_2Cl_2, 0°C; ii) NaN_3, DMF, 60°C, 71%; (b) i) BCl_3, CH_2Cl_2, -78°C, ii) BzCl, pyridine, 70%; (c) CF_3COCH_3, Oxone, $NaHCO_3$, MeCN/H_2O, **13**: 49%, **14**: 20%; (d) NaOMe, MeOH, 75%; (e) **15** or **16**, $CuSO_4$ (10 mol%), sodium ascorbate (15 mol%), Tol/*tert*-BuOH/H_2O, 90°C, MDW933 **5**: 56%, MDW941 **6**: 77%; (f) BCl_3, CH_2Cl_2, -78°C; (g) **15** or **16**, $CuSO_4$ (10 mol%), sodium ascorbate (15 mol%), DMF, MDW1064 **8**: 72%, MDW1065 **9**: 70%.

Determination of inhibition constants

Previously discontinuous methods have been used to determine the inhibition constants of glycosidase inhibitors. This method however proved to be unsuitable for the determination of the inhibition constants of KY170 **4**, MDW933 **5** and MDW941 **6** due to their high affinity/fast binding. Therefore the inhibition constants have been determined in a continuous substrate assay which was first described in 1982 (2) and has recently been reappraised in 2010 (3). In these experiments, inhibition of the enzyme and hydrolysis of the substrate proceed concurrently making the situation slightly more complex than the discontinuous (Scheme 2). The time-dependent interaction of inhibitor (I) with free β-glucosidase (E) was considered to occur in separate stages (A). A rapid reversible interaction is followed by a slower, irreversible reaction that transforms the reversible enzyme-inhibitor complex (EI) into an irreversible enzyme-inhibitor complex (EI*) (Supplementary Scheme 2). Progress curves were obtained (Supplementary Figure 1a) and apparent rate constant k' was plotted versus the concentration (Supplementary Figure 1b) to obtain estimates of the K_i (equilibrium constant) and k_i (rate constant)

Supplementary Scheme 2. Schematic representation of the process occurring during a continuous substrate assay.



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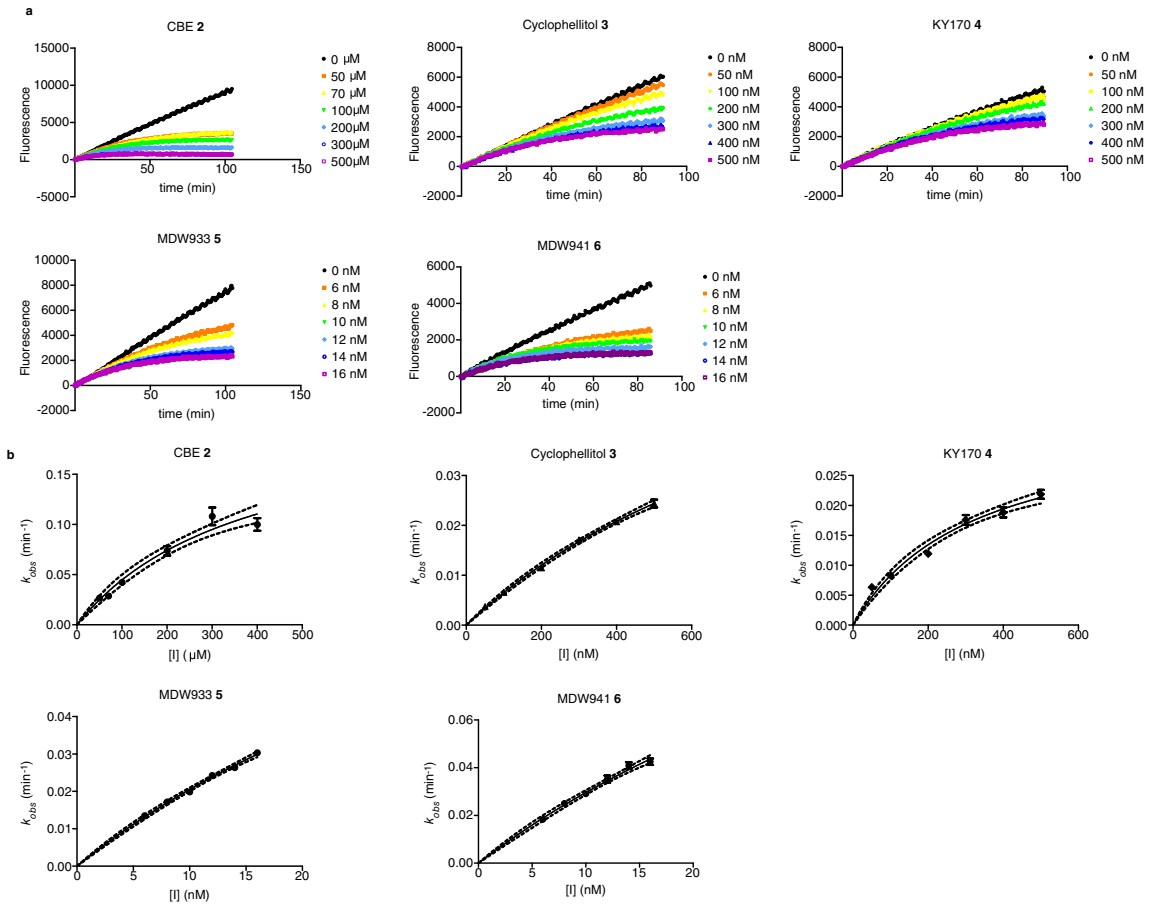

Supplementary Figure 1. Inhibition of GBA by probes. (A) Progress curves. An average of three individual measurements which is corrected for the blank and corrected to zero is represented. (B) *k'* versus $[I]_0$ plots. Data points represent mean of nine individual *k'* ± s.e.m. Solid lines represent best fit according to the described equitation. Dashed lines represent 95% intervals.

Molecular docking

Molecular docking was performed with KY170 **4**, MDW933 **5** and MDW941 **6** as ligands. MDW933 **5** and MDW941 **6** revealed to bind tightly to the GBA active site (Supplementary Figure 2). Both probes displayed free binding energies of ~8 kcal/mol. For both ligands we observed binding of the polar cyclophellitol head group in the active site pocket, with the epoxide being within 4 Angstrom of E340. The region immediately outside the active site pocket on the protein surface accommodated the hydrophobic tails. Inspection of binding showed tight binding of the hydrophobic part of the ligand

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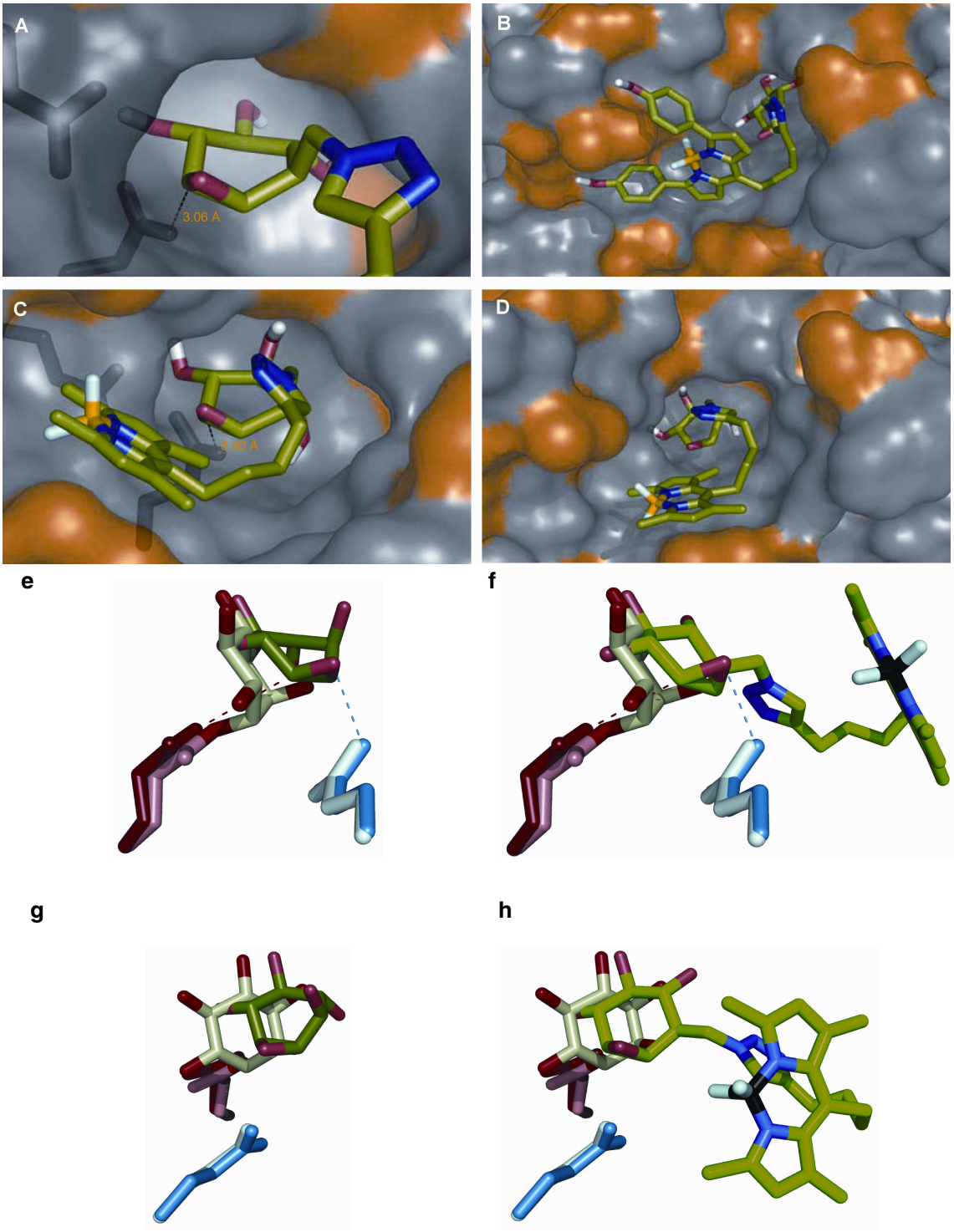
with this part of the protein. Binding of KY170 **4** was much weaker in the range of 5 kcal/mol, further illustrating how the hydrophobic tail greatly increased the binding affinity.



Supplementary Figure 2. Molecular docking of MDW933 and MDW941. Lowest energy conformers with rotatable bonds docked on crystal structure of glucocerebrosidase (pdb 2VE3).

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Inhibitor molecule is shown as sticks, GBA as semi-transparent space-filling model, and active-site residues E235 and E340 as dark-blue sticks (left and right, respectively) (A). Docking of MDW933 shows two common docking conformations (C, D) of -8.2 and -8.1 kcal/mol, docking of MDW941 resulted in -9.6 kcal/mol binding affinity (B). (E-H) Docking comparison with CBE-bound GBA (2VT0)**.** Top (E) and side (G) view of CBE docked on crystal structure 2V3E (E340, dark red; E235, dark blue; CBE, dark green) on top of covalent-bound CBE crystal structure 2VT0 (E340, pink; E235, light blue; CBE, pale green). Top (F) and side (H) view of an overlay of MDW933 (bright green).

Supplementary Figure 3. Mass spectrometric analysis of covalently labeled GBA. (A) Schematic representation of the experiment. Recombinant GBA labeled with KY170 **4** is digested with trypsin and analyzed by LC tandem MS (upper path). To release the label and to install the hydroxamic acid residue, GBA labeled with MDW933 **5** is treated with hydroxylamine prior to tryptic digestion and analysis by LC-MS/MS (lower path). KY170 **4** (B) and MDW933 **5** (C) are covalently linked to E340 in recombinant GBA. One peptide that was selected for fragmentation in the tryptic digest of GBA labeled with **4** had a parent mass (2071.99 amu) and one peptide that was selected for fragmentation in the tryptic digest of GBA labeled with **5** and treated with hydroxylamine had a parent mass (1885.92 amu). The mass of these peptides was 201.09 amu and 15.02 amu increased compared to the theoretic mass (1870.90 amu) of the sequence of the tryptic peptide containing residue E340 (shown in bold red). This is in accordance with the addition of the mass of respectively KY170 **4** (201.08 amu) and hydroxylamine (15.01 amu). Furthermore it is clear from the tandem mass spectra shown, that the adduct mass is localized at the glutamic acid residue of the peptide resulting in a delta-mass of 329.03 amu between y6, y7 and 330.14 amu between b10, b11, and a delta-mass of 144.03 amu between y6, y7 and 143.98 amu between b10, b11 for respectively KY170 **4** (B) and MDW933 **5** (C). This is consistent with the mass of glutamic acid covalently linked to KY170 **4** (330.12 amu) and hydroxylamine (144.05 amu) at that site. Ccam, cysteine residue with carbamidometyl modification.

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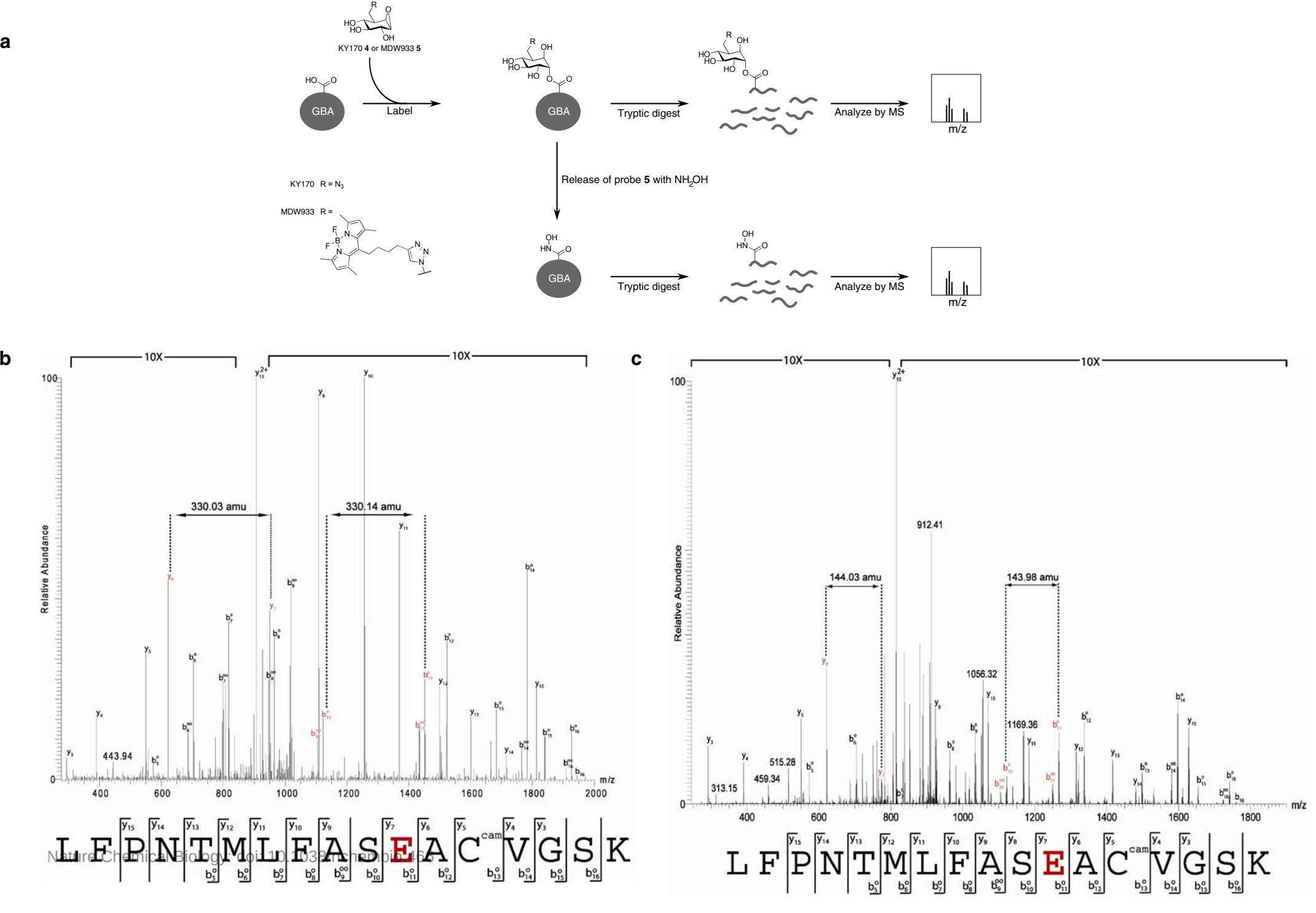
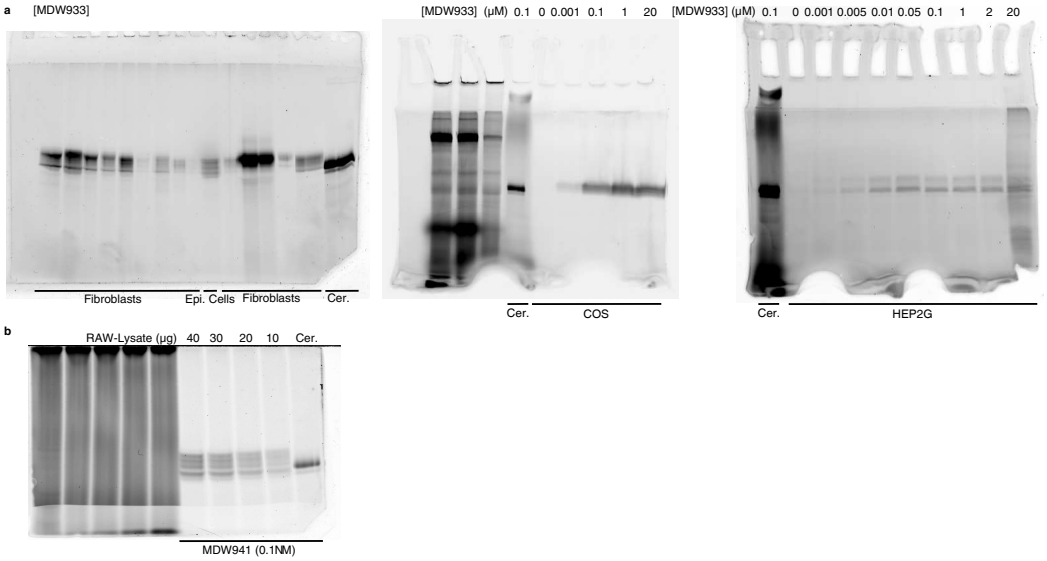
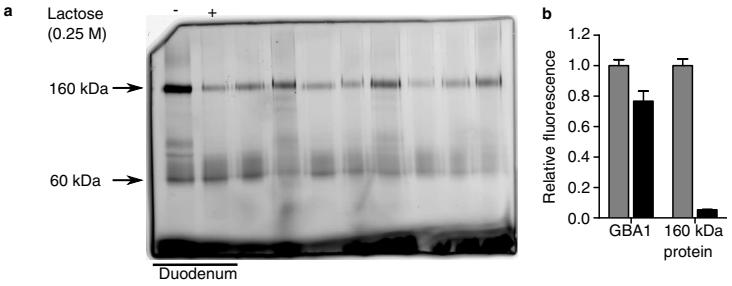




Supplementary Figure 4. Labeling of GBA in cell lysates. (**a**) MDW933 **5** (1 nM to 10 μM) selectively labeled GBA in fibroblast (left), COS cell lysate (middle) and HEP2G cell lysate (right). (**b**) Selective labeling of GBA in RAW-lysates (10-40 μg) using MDW941 **6** (0.1 μM).



Supplementary Figure 5. Indenfication of the 160 kDa protein. (a) Labeling of lactase by MDW933 **5** in homogenates of the duodenum of mice in the presence or absence of lactose (250 mM). (b) Quantification of the observed fluorescent signal. Data represent mean values ± s.d.

Cellular uptake of the probes

To identify the mechanism of entry of the probes, we performed a temperature experiment. We incubated cells with a serial dilution of MDW933 **5** either at 18°C, a temperature at which endocytosis is blocked, or 37°C for 2 hours. We harvested the cells and determined the

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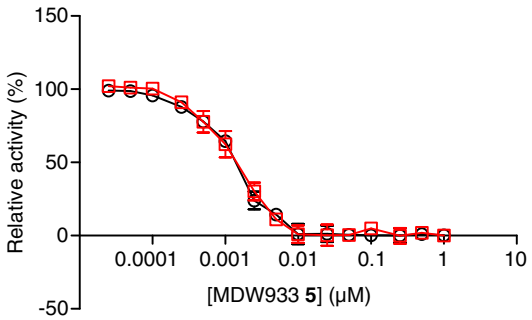
residual activity using a fluorogenic substrate assay. The residual activities showed that the temperature did not have an pronounced effect on *in situ* inhibition. At both temperatures, 10 nM MDW933 **5** blocked glucocerebrosidase activity *in situ* (SI Fig 6). We therefore exclude that the uptake is solely by endocytosis.



Supplementary Figure 6. In situ inhibition of GBA by MDW933 5 at 18°C (black line/circles) and 37°C (red line/squares). Data represent mean values ± s.d.

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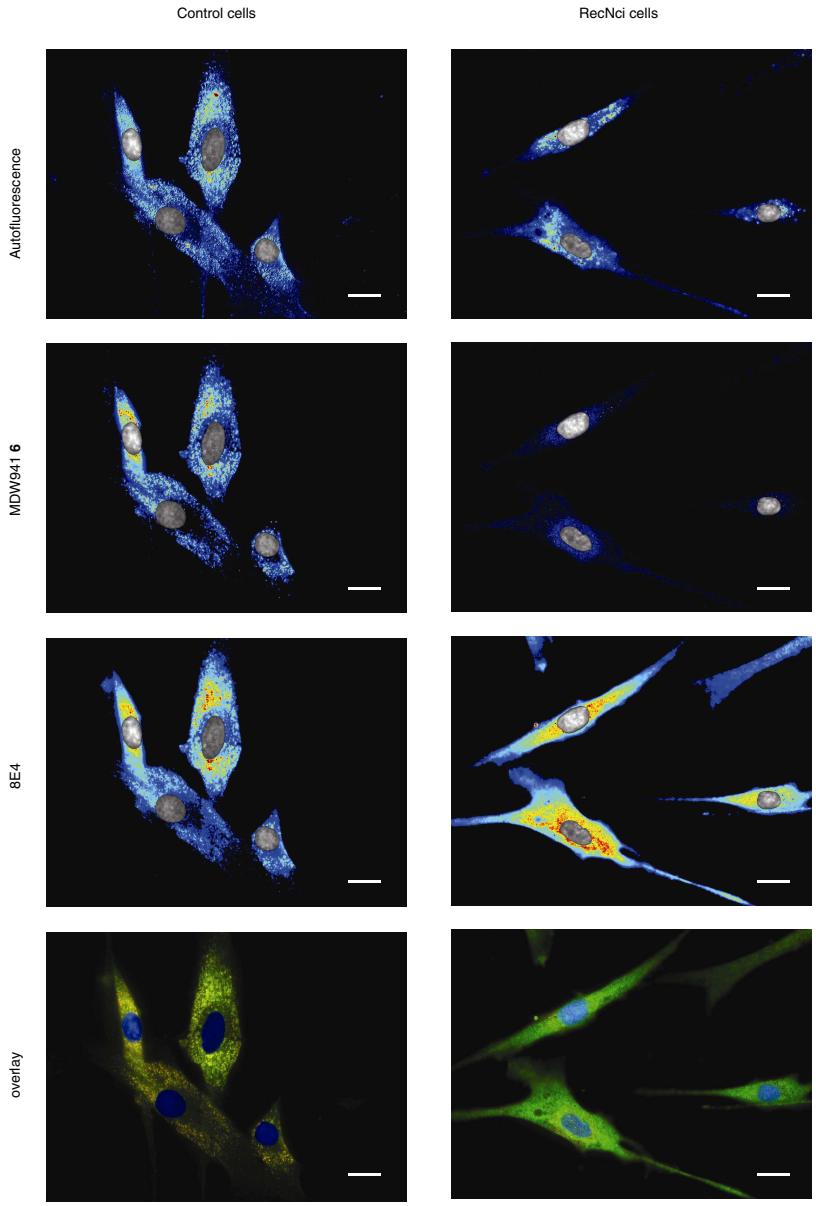

Supplementary Figure 7. Representative spectral imaging micrographs of control (left column) and gaucher (right column) cells labeled with MDW941 6. First row of panels represent a heat map of the autofluorescence. The second row of panels show a heat map of the observed BODIPY-fluorescence of GBA labeled with MDW941 **6** (unmixed and separated from the autofuorescence). The third row shows a heat map of the Alexa488-fluorescence of GBA visualized with monoclonal Ab 8E4 (unmixed and separated from the autofuorescence). In the fourth row an overlay of the unmixed and corrected signals of BODIPY and Alexa488 fluorescence is shown. Scale bar represents 20 μM.

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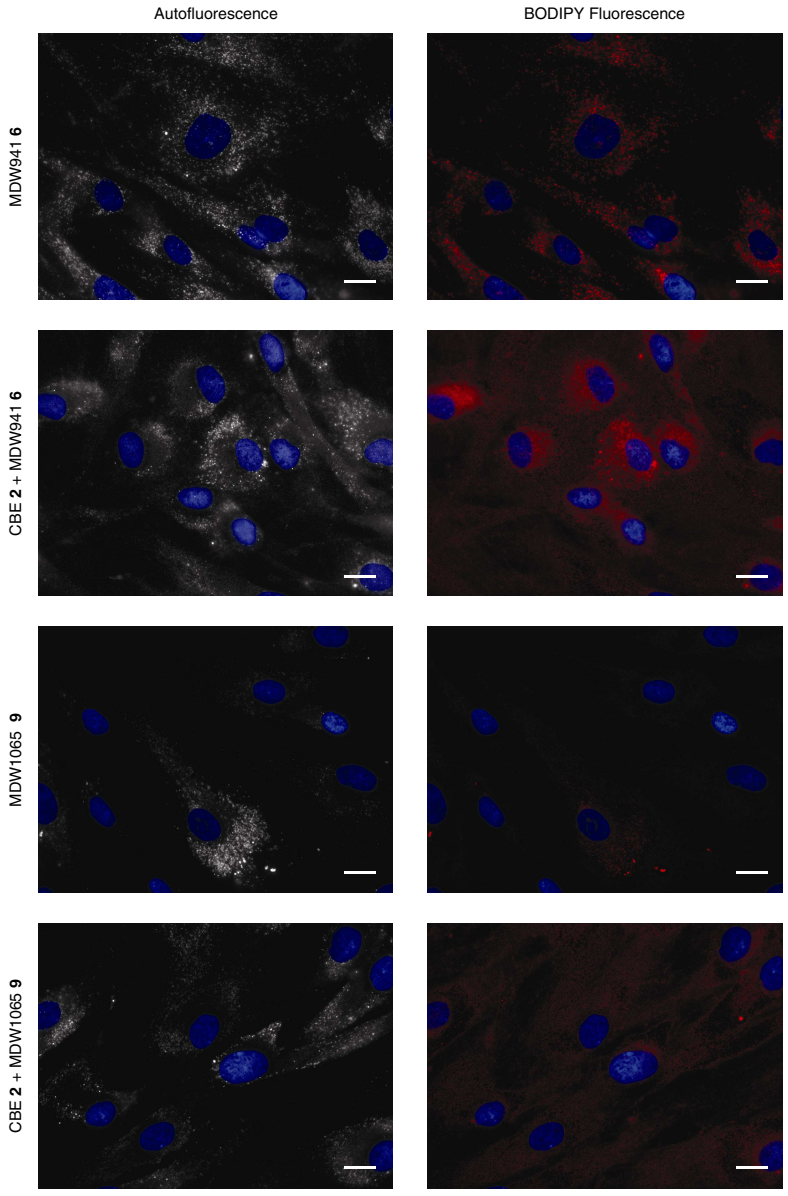

Supplementary Figure 8. Representative spectral imaging micrographs of cells labeled with MDW941 6 or control probe MDW1065 9 in the presence or absence of CBE 2. The left column shows the autofluorescence and the right column shows the unmixed BODIPY fluorescence signal. Upper row, micrographs of cells treated with MDW941 **6** in the absence of CBE **2** are shown. The second row of micrographs shows the cells treated with MDW941 **6** in the presence of CBE **2** (3 mM). The third row shows micrographs obtained after labeling cells with the non-reactive control probe MDW1065 **9**. In the final row, micrographs of cells treated with a combination of MDW1065 **9** and CBE **2** is shown. Scale bar represents 20 μM.

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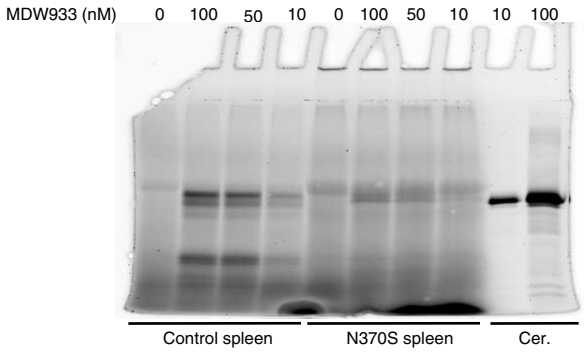
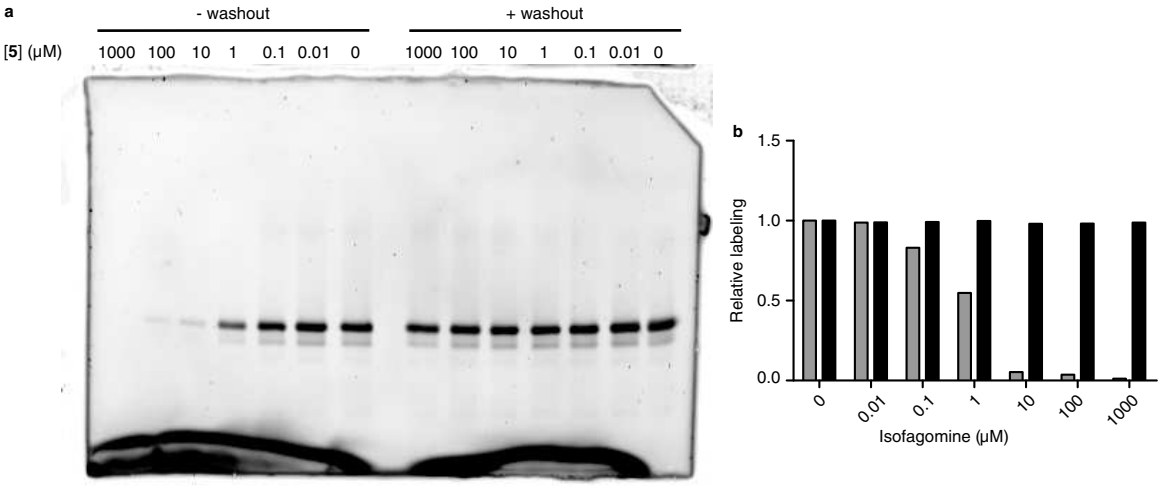


Supplementary Figure 9. Labeling of GBA in Gaucher and control spleen. Tissue homogenates were treated with 0, 10, 50 and 100 nM MDW933 **5** for 60 min.



Supplementary Figure 10. The reversibility of isofagomine inhibition. Recombinant GBA attached to monoclonal antibody 8E4 immobilized to Sepharose beads was pre-incubated for 15 min with increasing concentrations of isofagomine at pH 5.2 in the presence of taurocholate (0.2 % w/v) and Triton X-100 (0.1 % v/v). The bead suspension was washed with the same buffer or not and subsequently incubated for 15 min with 10 nM MDW933 **5**.

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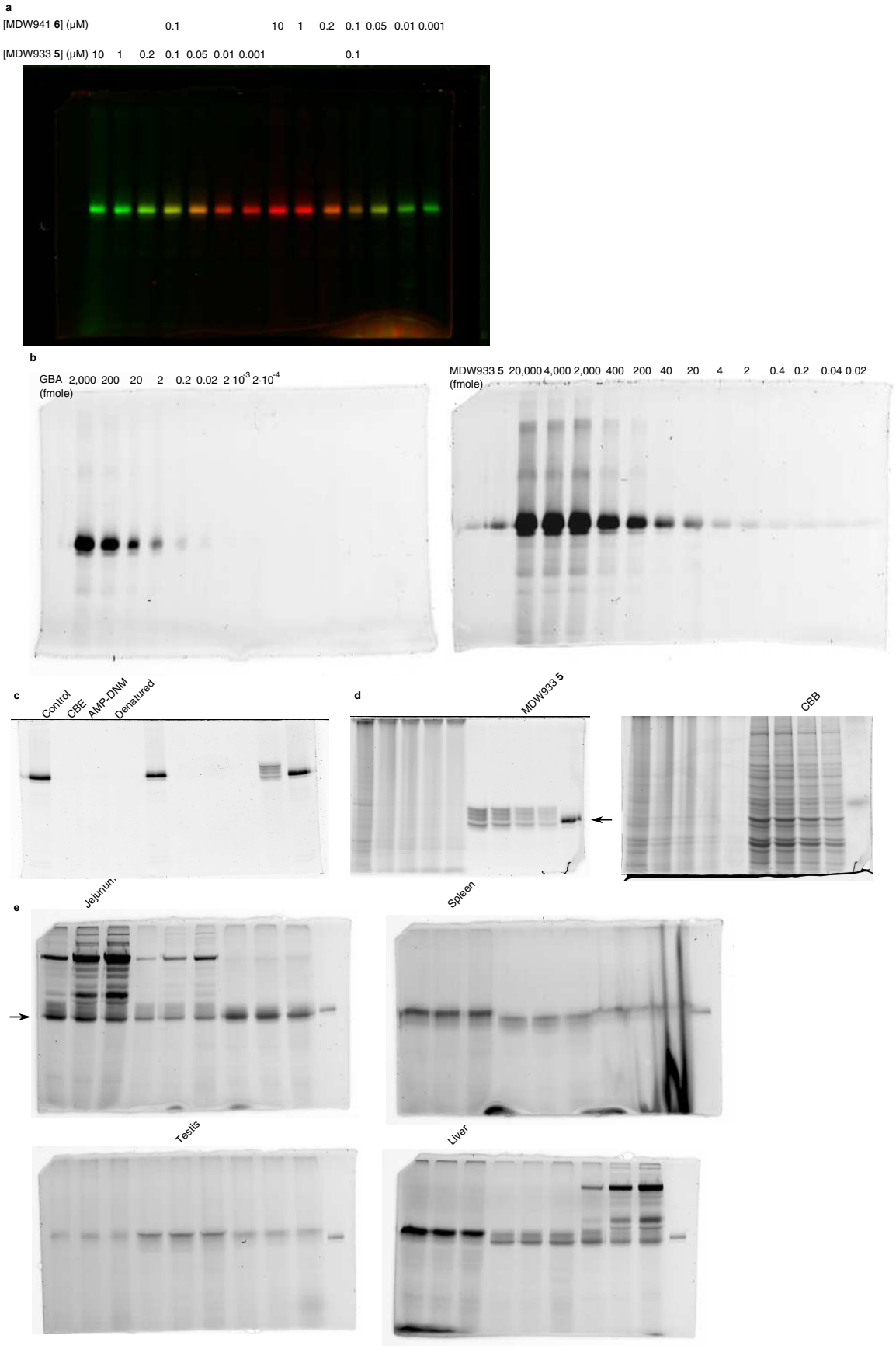

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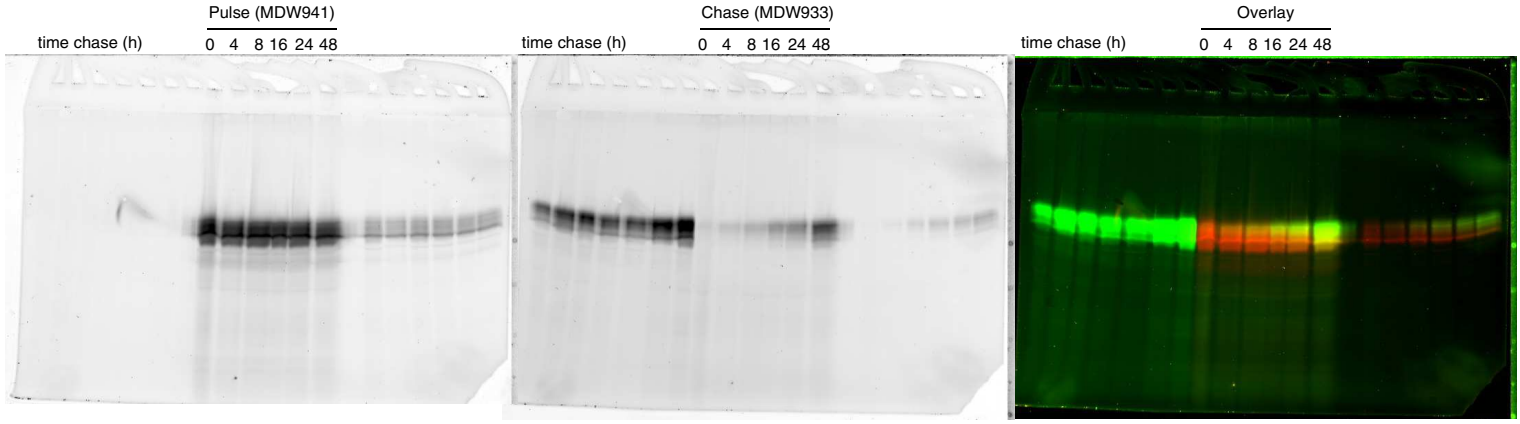
Supplementary Figure 11. Uncut gel images of Figure 2a, 2b, 2c, 2e and 2f are shown as respectively a, b, c, d, e.



Supplementary Figure 12. Uncut gel images of Figure 3d are shown.

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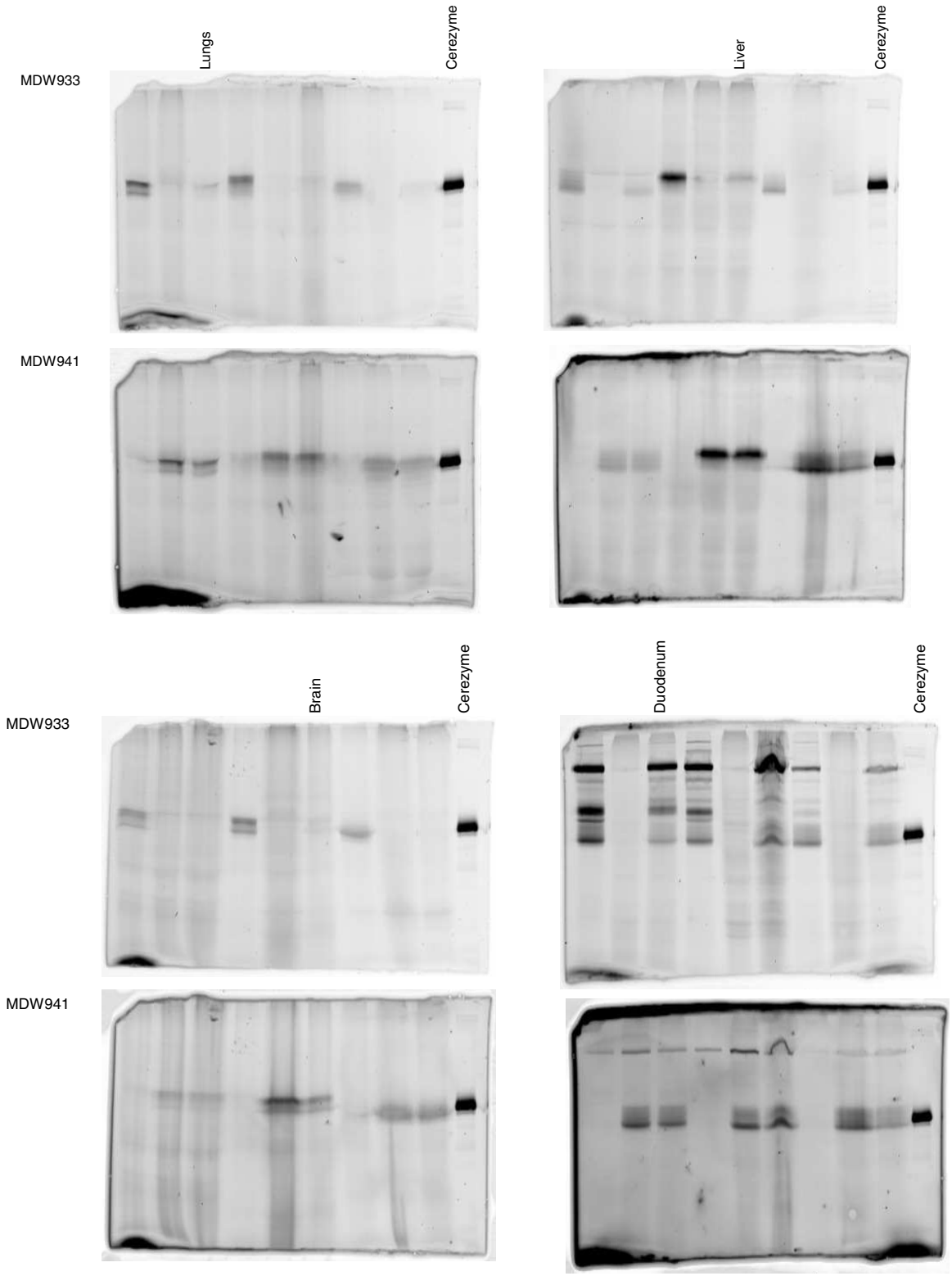

Supplementary Figure 13. Uncut gel images of Figure 4a are shown.

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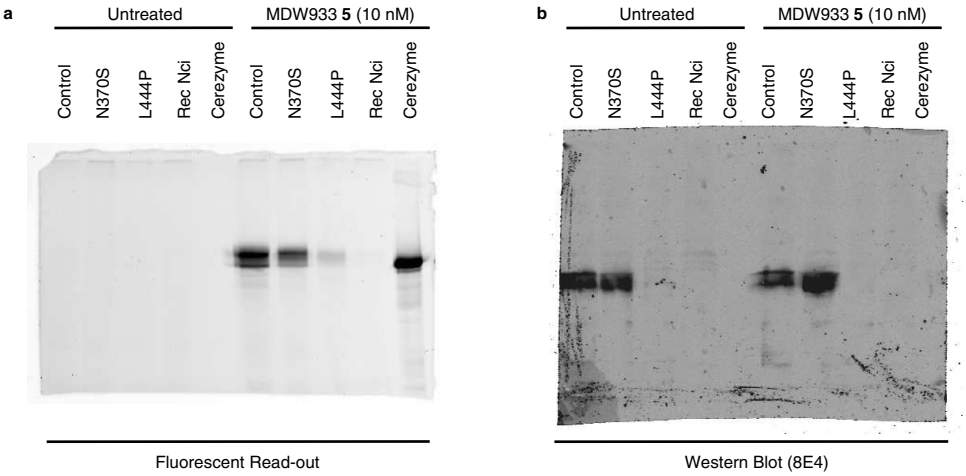

Supplementary Figure 14. Uncut gel images of Figure 5a. Left: fluorescent read-out. Right: GBA is visualized using Western blotting

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Supplementary methods

All reagents were of a commercial grade and were used as received unless stated otherwise. Isofagomine **1** (4), cyclophellitol **3** (1) and BODIPY alkyne green **15** and red **16** (5) were synthesized as described in literature and their spectral data was in accordance with those reported in literature. The AMP-DNM **7** used in this research was from a previously synthesized batch (6). Diethyl ether (Et_2O), ethyl acetate (EtOAc), light petroleum ether and toluene were obtained from Riedel-de Haën. Acetonitrile, dichloromethane, dimethylformamide (DMF), methanol (MeOH), pyridine, tetrahydrofuran (THF) were purchased from Biosolve. Dichloromethane was distilled from CaH_2 and THF was distilled over $LiAlH_4$ prior to use. All reactions were performed under an inert atmosphere or Argon unless stated otherwise. Solvents used for flash chromatography were of pro analysi quality. Reactions were monitored by TLC analysis using Merck aluminum sheets precoated with silica gel 60 with detection by UV-absorption (254 nm) and by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot H_2O$ (10 g/L) in 10% aqueous sulfuric acid followed by charring at ~150°C or by spraying with 20% sulfuric acid in ethanol followed by charring at ~150°C. Column chromatography was performed using either Baker- or Screening Device silica gel in the indicated solvents. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DMX-400 (400/100 MHz) or a Bruker AV-400 (400/100 MHz) spectrometer in the given solvent. Chemical shifts are reported as δ-values in ppm relative to the chloroform residual solvent peak or tetramethylsilane (TMS) as internal standard. Coupling constants are given in Hz. All given ^{13}C spectra are proton decoupled. Spin multiplicities are given as s, d, dd, ddd, dddd, dt, t, td, q and m. High resolution mass spectra were recorded with a LTQ Orbitrap (Thermo Finnigan). LC/MS analysis was performed on a Jasco HPLC-system (detection simultaneously at 214 nm and 254 nm) equipped with an analytical Alltima C18 column (Alltech, 4.6 mmD × 250 mmL, 5μ particle size) in combination with buffers A: H_2O, B: acetonitrile and C: 1% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument. Optical rotations were measured on a Propol automatic polarimeter (sodium D line, λ = 589 nm). FT-IR-spectra were recorded on a Paragon-PE 1000.

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(1R,2R,5S,6S)-2-(azidomethyl)-5,6-bis(benzyloxy)cyclohex-3-enol (11)

To a solution of **10** (1.24 g, 3.65 mmol) in dichloromethane (26 mL) were added *p*-toluenesulfonylchloride (1.04 g, 5.48 mmol, 1.1 equiv.) and triethylamine (0.90 mL, 6.57 mmol, 1.8 equiv.) at 0°C. The solution was stirred for 5 h before being poured in 1M HCl solution. The mixture was extracted with Et_2O and the organic layer was dried over $MgSO_4$ before being concentrated *in vacuo*, yielding the crude tosylate which was immediately subjected to azidation. To a solution of tosylated intermediate (1.75 g, 3.65 mmol) in DMF (35 ml) was added sodium azide (2.40 g, 36.7 mmol, 10.4 equiv.). The solution was stirred for 24 h at 60°C before being concentrated *in vacuo*. The crude product was diluted with EtOAc, washed with 1 M HCl, saturated aqueous $NaHCO_3$ and brine. The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Purification by silica column chromatography (8%→16% EtOAc in petroleum ether) afforded **11** (900 mg, 2.46 mmol, 71%) as an amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33-7.26 (m, 10H), 5.79 (dt, J=10.4, 2.4 Hz, 1H), 5.58 (dt, J=10.4, 2.4 Hz, 1H), 5.02 (d, J=11.3, 1H), 4.7 (dd, J=11.2, 5.4 Hz, 2H), 4.65 (d, J=11.2 Hz, 1H), 4.21-4.19 (m, 1H), 3.61-3.53 (m, 3H), 3.44 (dd, J=12.0, 6.0 Hz, 1H), 2.83 (s, 1H) and 2.48 (br, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.1, 137.2, 128.7, 128.6, 128.0, 127.9, 127.8, 127.7, 83.5, 80.3, 75.0, 71.6, 52.5 and 43.6. FT-IR: ν_{max} (neat)/cm^{-1}: 2095.9, 1497.1, 1453.9, 1275.9, 1092.6, 1050.4, 1027.7, 732.0 and 695.9. $[\alpha]_D^{20}$ +137.8° (c=1, $CHCl_3$). LC/MS: R_t 9.35; linear gradient 10→90% B in 13.5 min; ESI/MS: m/z=383.1 $[M+NH_4]^+$. HRMS: ($M+3H^+-N_2$) calcd for $C_{21}H_{26}NO_3$ 340.19072 found 340.19080.

(1R,2R,3S,6R)-6-(azidomethyl)cyclohex-4-ene-1,2,3-triyl tribenzoate (12)

Borontrichloride (21 mL, 1M in CH_2Cl_2, 21.1 mmol, 10 equiv.) was added to a solution of **11** (777.1 mg, 2.11 mmol) in anhydrous dichloromethane (10 mL) at -78°C. The reaction mixture was stirred at -78°C for 6 h before being quenched with MeOH. The solution was concentrated *in vacuo* giving the triol intermediate, which was immediately used for benzoylation. The crude product was coevaporated several times with anhydrous toluene before being dissolved in pyridine (10 mL). Benzoylchloride (2.6 mL, 21.1 mmol, 10 equiv.) was added at 0°C and the reaction mixture was stirred for 18 h at

ambient temperature. The mixture was quenched with saturated aqueous $NaHCO_3$, extracted with EtOAc, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by silica column chromatography (4%→6% EtOAc in petroleum ether) afforded **12** (701.8 mg, 1.46 mmol, 70%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (d, J=7.2 Hz, 2H), 7.92 (d, J=7.2 Hz, 2H), 7.84 (d, J=7.2 Hz, 2H), 7.53-7.46 (m, 3H), 7.40 (dt, J=24.4, 8.0 Hz, 5H), 7.26-7.18 (m, 2H), 6.00-5.93 (m, 3H), 5.86 (d, J=10.0 Hz, 1H), 5.72 (t, J=9.2 Hz, 1H), 3.64 (dd, J=12.4, 4.0 Hz, 1H), 3.46 (dd, J=12.4, 6.4 Hz, 1H) and 2.99-2.97 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.0, 165.9, 133.3, 133.2, 133.1, 129.8, 129.7, 129.6, 129.4, 129.0, 128.9, 128.5, 128.4, 128.3, 126.2, 127.0, 72.7, 72.7, 72.6, 70.4, 52.0 and 42.5. FT-IR: ν_{max} (neat)/cm^{-1}: 2100.3, 1718.0, 1601.8, 1585.4, 1492.2, 1314.5, 1250.8, 1178.0, 1108.8, 1031.8, 1025.9, 950.7, 855.3, 778.1, 705.0 and 686.1. $[\alpha]_D^{20}$ +173° (c=1.0, $CHCl_3$). LC/MS: R_t 10.68; linear gradient 10→90% B in 13.5 min; ESI/MS: m/z = 498.2 $[M+H]^+$. HRMS: ($M+Na^+$) calcd for $C_{28}H_{25}NO_6$ 520.14791 found 520.14724.

(2S,3R,4S,5S)-2,3,4-Benzoyl-8-azido-8-deoxy-cyclophellitol (13 and 14)

A solution of 0.4 mM Na_2EDTA solution in H_2O (3.1 mL) and trifluoroacetone (1.34 mL, 15 mmol, 15 equiv.) were added to **12** (497 mg, 1.0 mmol) in acetonitrile (6.7 mL). A mixture of oxone (3.07 g, 5.0 mmol, 5 equiv.) and $NaHCO_3$ (588.1 mg, 7.0 mmol, 7 equiv.) was added to the solution over a period of 15 min. After stirring at 4°C for 4 h, an additional amount of 0.4 mM Na_2EDTA in H_2O (1.5 mL), trifluoroacetone (0.7 mL, 7.5 mmol, 7.5 equiv.) and a mixture of oxone (1.5 g, 2.5 mmol, 2.5 equiv.) and $NaHCO_3$ (290 mg, 3.5 mmol, 3.5 equiv.) were added to the reaction mixture over a period of 15 min. The reaction mixture was stirred at 4°C for 30 min before being diluted with H_2O. After extraction of the water layer with EtOAc, the combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Purification by silica column chromatography (8%→10% Et_2O in petroleum ether) and (16%→18% Et_2O in petroleum ether) afforded **13** (103.9 mg, 0.20 mmol, 20%) and **14** (253.7 mg, 0.49 mmol, 49%) respectively as amorphous solid.

14: ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (d, J=7.4 Hz, 2H), 7.88 (d, J=7.6 Hz, 2H), 7.79 (d, J=7.6 Hz, 2H), 7.56 (t, J=7.2 Hz, 1H), 7.46-7.36 (m, 5H), 7.32 (t, J=7.6 Hz, 2H), 7.24 (t, J=7.2, 2H), 5.84 (t,

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J=9.2 Hz, 1H), 5.56 (d, J=8.8 Hz, 1H), 5.43 (t, J=10.0 Hz, 1H), 3.67-3.62 (m, 4H), 3.44 (s, 1H) and 2.71 (dddd, J=9.3, 7.6, 1.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.7, 165.6, 165.4, 133.5, 133.3, 133.1, 129.8, 129.6, 129.5, 128.9, 128.7, 128.6, 128.4, 128.3, 128.1, 72.2, 71.4, 67.8, 54.7, 54.2, 50.5 and 40.8. FT-IR: ν_{max} (neat)/cm^{-1}: 2104.5, 1722.6, 1601.9, 1451.6, 1315.2, 1258.3, 1178.4, 1094.8, 1069.6, 1026.1, 853.8, 706.6 and 686.1. $[\alpha]_D^{20}$ + 93.6° (c=1.0, $CHCl_3$). LC/MS: R_t 10.24; linear gradient 10→90% B in 13.5 min; ESI/MS: m/z = 514.2 $[M+H]^+$. HRMS: ($M+H^+$) calcd for $C_{28}H_{23}N_3O_7$ 514.16088 found 514.16007.

13: ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (d, J=7.2 Hz, 2H), 7.89 (d, J=7.2 Hz, 2H), 7.78 (d, J=7.2 Hz, 2H), 7.53-7.19 (m, 5H), 5.96 (t, J=9.6 Hz, 1H), 5.77 (d, J=8.8 Hz, 1H), 5.55 (t, J=9.6 Hz, 1H), 3.77-3.74 (m, 2H), 3.64 (dd, J=12.8, 4.0 Hz, 1H), 3.32 (s, 1H) and 2.68 (ddd, J=9.2, 5.2, 3.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.0, 165.9, 165.6, 133.4, 133.0, 129.9, 129.7, 129.5, 129.0, 128.9, 128.6, 128.4, 128.3, 128.1, 72.1, 70.0, 69.9, 54.6, 53.8, 50.8 and 40.9. FT-IR: ν_{max} (neat)/cm^{-1}: 2104.6, 1717.8, 1602.1, 1451.8, 1249.4, 1178.1, 1093.3, 1069.0, 1026.0 and 704.5. $[\alpha]_D^{20}$ +52.4° (c=1.0, $CHCl_3$). LC/MS: R_t 10.22; linear gradient 10→90% B in 13.5 min; ESI/MS: m/z = 514.2 $[M+H]^+$. HRMS: ($M+H^+$) calcd for $C_{28}H_{23}N_3O_7$ 514.16088 found 514.16017.

(2S,3R,4S,5S)-8-azido-8-deoxy-cyclophellitol (KY170, 4)

A catalytic amount of NaOMe was added to a solution of **14** (103.9 mg, 0.20 mmol) in MeOH (1.0 mL) and stirred for 1 h at ambient temperature. The reaction mixture was neutralized with Amberlite IR-120 H^+, filtered and concentrated *in vacuo*. Purification by silica column chromatrography (6%→8% MeOH in dichloromethane) provided KY170 **4** (30.0 mg, 0.15 mmol, 75%). ^{1}H NMR (400 MHz, MeOD): δ 3.84 (dd, J=8.4, 3.6 Hz, 1H), 3.67 (d, J=8.0 Hz, 1H), 3.51 (dd, J=12.0, 8.8 Hz, 1H), 3.36 (d, J=3.2 Hz, 1H), 3.23 (dd, J=10.0, 8.4 Hz, 1H), 3.13-3.08 (m, 2H), 2.07 (ddt, J=9.4, 3.6, 1.6 Hz, 1H). ^{13}C NMR (100 MHz, MeOD): δ 78.3, 72.7, 68.6, 57.6, 56.1, 52.4 and 43.9. FT-IR: ν_{max} (neat)/cm^{-1}: 3331.7, 3187.9, 2936.1, 2097.6, 1455.8, 1345.9, 1273.4, 1144.2, 1092.5, 1066.5, 1032.1, 995.1, 926.6, 899.8, 818.7, 803.5, 714.1 and 652.0. $[\alpha]_D^{20}$ + 174.7° (c=0.6, MeOH). LC/MS: R_t 0.95; linear

gradient 10→90% B in 13.5 min; ESI/MS: m/z = 219.2 $[M+NH_4]^+$. HRMS: $(M+3H^+-N_2)$ calcd for $C_7H_{14}NO_4$ 176.09173 found 176.09179.

MDW933 5

KY170 **4** (8.51 mg, 42 μmol) and BODIPY Green-alkyne **15** (13.8 mg, 42 μmol) (5) were dissolved in tert-BuOH/Tol/H_2O (1.8 mL, 1/1/1 v/v/v). $CuSO_4$ (100 mM in H_2O, 42 μL, 4.2 μmol) and sodium ascorbate (100 mM in H_2O, 63 μL, 6.3 μmol) were added. Subsequently, the reaction was heated to 80°C and stirred overnight. The solution was diluted with CH_2Cl_2, washed with H_2O, dried and concentrated. Purification over silica gel column chromatography (0%→5% MeOH/CH_2Cl_2) gave MDW933 **5** as an orange powder (56%, 12.49 mg, 23.6 μmol).^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.40 (s, 1H), 6.01 (s, 2H), 4.68 (d, J = 12.0 Hz, 1H), 4.58 (dd, J = 13.4, 7.5 Hz, 1H), 3.66 (d, J = 5.6 Hz, 1H), 3.40-3.34 (m, 1H), 3.20-3.15 (m, 1H), 3.02 (s, 1H), 2.97 (s, 1H), 2.96-2.91 (m, 2H), 2.73 (t, J = 6.4, 6.4 Hz, 2H), 2.49-2.46 (s, 6H), 2.45-2.40 (m, 1H), 2.33 (s, 6H), 1.86 (td, J = 15.0, 7.6, 7.6 Hz, 2H), 1.66-1.58 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 153.9, 146.0, 140.3, 131.4, 121.7, 77.2, 77.0, 76.9, 76.7, 71.1, 67.4, 56.0, 54.5, 49.6, 43.0, 31.2, 29.5, 28.1, 25.2, 16.3, 14.4. LC/MS: Rt 6.83; linear gradient 10→90% B in 13.5 min; ESI/MS: m/z = 530.00 $[M+H]^+$. HRMS: $(M+H^+)$ calcd for $C_{26}H_{34}BF_2N_5O_4$ 530.27447 found 530.27454

MDW941 6

KY170 **4** (5.46 mg, 27 μmol) and BODIPY Red-alkyne **16** (13.1 mg, 27 μmol) (5) were dissolved in *tert*-BuOH/Tol/H_2O (1.5 mL, 1/1/1 v/v/v). $CuSO_4$ (100 mM in H_2O, 27 μL, 2.7 μmol) and sodium ascorbate (100 mM in H_2O, 41 μL, 4.1 μmol) were added. Subsequently, the reaction was heated to 80°C and stirred overnight. The solution was diluted with CH_2Cl_2, washed with H_2O, dried and concentrated. Purification over silica gel column chromatography (0%→5% MeOH in dichloromethane) gave MDW941 **6** as an purple powder (77%, 14.32 mg, 20.8 μmol).^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.80 (d, J = 8.5 Hz, 4H), 7.36 (s, 1H), 7.17 (d, J = 3.4 Hz, 2H), 6.89 (d, J = 8.5 Hz, 4H), 6.54 (d, J = 3.8 Hz, 2H), 5.12-4.83 (m, 1H), 4.80-4.46 (m, 3H), 3.77 (s, 6H), 3.75-3.68 (m,

1H), 3.45-3.34 (m, 1H), 3.26-3.13 (m, 1H), 3.11-3.02 (m, 1H), 3.00-2.94 (m, 1H), 2.90-2.78 (m, 2H), 2.73-2.58 (m, 2H), 2.51-2.36 (m, 1H), 2.08-1.91 (m, 2H), 1.84-1.67 (m, 4H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 160.5, 157.4, 144.6, 136.0, 130.9, 126.8, 125.0, 120.0, 113.7, 71.0, 67.2, 56.0, 55.2, 54.6, 49.6, 42.7, 33.0, 30.3, 29.7, 29.4, 25.0. LC/MS: Rt 8.35; linear gradient 10→90% B in 13.5 min; ESI/MS: m/z = 686.07 $[M+H]^+$. HRMS: $(M+H^+)$ calcd for $C_{36}H_{38}BF_2N_5O_4$ 686.29560 found 686.29559.

(1R,2R,3S,6R)-6-(azidomethyl)cyclohex-4-ene-1,2,3-triol (17)

Azide **11** (103 mg, 0.28 mmol) was dissolved in CH_2Cl_2 (5 mL) and cooled to -78ºC before BCl_3 (5 mL, 1M in CH_2Cl_2) was added. After 4 h stirring at -78°C, the reaction was quenched by the addition of MeOH. The temperature was raised to room temperature and the solution was concentrated *in vacuo*. Trace of BCl_3 were removed by coevaporating with MeOH. The resulting crude triol **17** was used as such in the click reaction.

MDW1064 (8)

Azide **17** (37 mg, 0.15 mmol) was dissolved in DMF (2 mL). To the solution was added BODIPY Green-alkyne (82 mg, 0.25 mmol), $CuSO_4$ (1M in H_2O, 15 µL, 15 µmol) and sodium ascorbate (1M in H_2O, 23 µL, 23 µmol). The reaction was stirred overnight, after which TLC analysis revealed complete conversion. The solution was diluted with EtOAc, washed with 1M HCl, $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure. Silica gel column chromatography (CH_2Cl_2→5% MeOH/CH_2Cl_2) afforded MDW1064 **8** as an orange amorphous solid (72%, 55.3 mg, 108 µmol). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ ppm 7.48 (s, 1H), 6.08 (s, 2H), 5.58 (td, *J* = 10.2, 2.3, 2.3 Hz, 1H), 5.43 (td, *J* = 10.4, 2.0, 2.0 Hz, 1H), 4.61 (dd, *J* = 13.8, 3.8 Hz, 1H), 4.47 (dd, *J* = 13.9, 7.3 Hz, 1H), 3.99-3.95 (m, 1H), 3.47 (dd, *J* = 9.9, 8.0 Hz, 1H), 3.30 (t, *J* = 9.7, 9.7 Hz, 1H), 3.03-2.98 (m, 2H), 2.78 (t, *J* = 7.5, 7.5 Hz, 2H), 2.75-2.67 (m, 1H), 2.49 (s, 6H), 2.40 (s, 6H), 1.95-1.86 (m, 2H), 1.74-1.64 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$/MeOD) δ ppm 153.4, 146.8, 145.7,

140.2, 130.9, 130.5, 125.0, 121.9, 121.3, 76.8, 71.6, 70.3, 50.6, 44.0, 30.9, 29.2, 27.6, 24.7, 15.7, 13.7. LC/MS: R_t 7.22; linear gradient 10→90% B in 13.5 min; ESI/MS: m/z=513.87 $[M+H]^+$.

MDW1065 (9)

Azide **17** (33 mg, 0.15 mmol) was converted to the title compound as described above. To the solution was added BODIPY Red-alkyne (100 mg, 0.25 mmol), $CuSO_4$ (1M in H_2O, 15 μL, 15 μmol) and sodium ascorbate (1M in H_2O, 23 μL, 23 μmol). The reaction was stirred overnight, after which TLC analysis revealed complete conversion. The solution was diluted with EtOAc, washed with 1M HCl, $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure. Silica gel column chromatography (CH_2Cl_2→5% MeOH/CH_2Cl_2) afforded title MDW1065 **9** as a purple solid (70%, 71 mg, 106 μmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.89-7.78 (m, 4H), 7.36-7.27 (m, 1H), 7.27-7.21 (m, 2H), 6.96-6.89 (m, 4H), 6.62-6.56 (m, 2H), 5.61-5.53 (m, 1H), 5.48-5.36 (m, 1H), 4.54-4.45 (m, 2H), 4.01-3.93 (m, 1H), 3.86-3.79 (m, 6H), 3.50 (t, *J* = 8.8, 8.8 Hz, 1H), 3.29 (t, *J* = 9.6, 9.6 Hz, 1H), 3.00-2.87 (m, 2H), 2.81-2.65 (m, 2H), 2.63-2.48 (m, 1H), 1.91-1.75 (m, 4H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 160.4, 157.4, 147.2, 144.6, 135.9, 130.8, 126.7, 125.4, 125.0, 122.0, 119.9, 113.6, 77.1, 71.8, 70.3, 55.1, 50.8, 44.3, 32.9, 30.2, 29.2, 24.9. LC/MS: R_t 8.36; linear gradient 10→90% B in 13.5 min; ESI/MS: m/z=670.00 $[M+H]^+$.

Determination of the binding constants

To 100 μL McIlvaine buffer (pH 5.2, 0.2% sodium taurocholate, 0.1% Triton X-100) containing 4-methylumbelliferyl β-D-glucoside (7.5 mM) in Greiner flat bottom black 96-well plate was added 5 μL inhibitor (25× stock in DMSO). The resulting mixture was preincubated at 37°C for 15 min in a TECAN GENios microplate reader. GBA (2 ng) in McIlvaine buffer (20 μL pH 5.2, 0.2% sodium taurocholate, 0.1% Triton X-100) was preheated to 37°C for 15 min before being added to the substrate mix. The resulting solution was mixed by horizontal shaking for 15 sec, after which release 4-methylumbelliferyl measured with a TECAN GENios platereader (λ_{ex} 340 nm and λ_{em} 465 nm) for the indicated time. Bleaching of the fluorophore, which was observed during the assay, was corrected

by subtracting blanks (100 µL substrate buffer, 5 µL DMSO, 20 µL McIlvaine buffer). Apparent rate constants *k'* were obtained by fitting the resulting progress curves (Figure 1A) to the one-phase association equitation $y = (y_{max} - y_0)(1 - \exp^{-k't}) + y_0$ in GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Measurements were performed in nine fold and plotting of the obtained *k'* values versus $[I_0]$ yielded rectangular hyperbolar functions (Figure 1B). Using the equitation $k' = \frac{k_i[I_0]}{K_{i\,app} + [I_0]}$ estimates of $K_{i\ app}$ and k_i were obtained. The K_i value in the absence of substrate was obtained by correcting the $K_{i\ app}$ with $(1 + \frac{[S]}{K_m})$. The K_m of 4-methylumbelliferyl β-D-glucoside for GBA is 1.2 mM.

Molecular docking

KY170 **4**, MDW933 **5**, and MDW941 **6** were docked on GBA as follows. Allowing conformational changes in the small compounds, enzyme-ligand complexes were simulated with a low free energy. Ligand molecules were prepared using MarvinSketch. Lowest energy conformers were calculated and saved as .pdb files that were used as input for AutodockTools. Rotatable bonds were defined and .pdbqt files were generated that served as input for Autodock Vina (7). Similarly, protein .pdbqt files were also prepared using AutodockTools. The protein file was produced from the crystal structure coordinates of glucocerebrosidase in the open state (pdb 2VE3,(8)). Autodock Vina experiments were run on a 2.66 GHz Intel duo core iMac computer (Apple Macintosh). Flexible docking experiments were run using standard settings apart from the exhaustiveness, which was set at 10. A search space spanning roughly 30 cubic Ångströms around the active site was defined.

Time-lapse microscopy

Fibroblasts were cultured in chamber slides (Lab-Tek II, Nunc, Roskilde, Denmark) and incubated with 5 nM compound MDW933 **5** or MDW941 **6**; immediately after which mineral oil was thinly layered on top of the culture medium to prevent evaporation. With the 37°C, 10% CO_2 incubator enclosing the microscope, cells were imaged every 5 min for 2 hours simultaneously using

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fluorescence microscopy with an N2.1 filter block and phase-contrast brightfield microscopy (Leica IR-BE with Z-motor drive and a Plan APO 63×/1.40 oil immersion objective (Leica Microsystems, Rijswijk, The Netherlands), equipped with a KX85 camera, Apogee Instruments, Auburn, CA, USA). To minimize photo-toxicity fluorescence imaging was limited to 2 h; to monitor for possible toxicity due to the presence of MDW933 **5** or MDW941 **6**, after 2 h live-cell imaging was continued with bright field microscopy only for another 98 hours. An auto-focus routine was applied during acquisition. Images were analyzed using TimeLapseAVI 5.1.4 software (©Ron Hoebe, CMO, AMC, University of Amsterdam, The Netherlands; R.A.Hoebe@amc.uva.nl).

Mass spectrometric analysis of GBA labeled with KY170 4 and MDW933 5

Identification of the site of binding of KY170 4

GBA (5 μg) was labeled with 6 μM KY170 **4** in McIlvaine buffer (150 mM, pH 5.2, 0.2% sodium taurocholate (w/v), 0.1% Triton X-100 (v/v)) for 1h at 37°C (10 μL end volume). Prior to digestion the pH was adjusted by the addition of 200 mM NH_4HCO_3 (pH 8.0), and the protein was reduced with 10 mM dithiothreitol for 30 min at 60°C and alkylated with 15 mM iodoacetamide for 30 min at RT in the dark. The labeled protein was digested by addition of 50 ng trypsin (sequencing grade modified, Promega) and incubation at 37°C overnight. Protein digests were desalted using reversed phase C_{18}-Ziptips (2 μg capacity, Millipore). After activation in acetonitrile and loading of the samples, the Ziptips were washed with 0.1% aqueous trifluoroacetic acid and eluted with 60% acetonitrile, 0.1% trifluoroacetic acid and 39.9% water. Prior to loading onto the LC-MS system samples were diluted 10-fold with 0.1% aqueous trifluoroacetic acid.

Identification of the site of binding of MDW933

Some 40 μg of recombinant GBA (1 μg/μL) was diluted in 60 μL McIlvaine buffer (150 mM, pH 5.2, 0.2% sodium taurocholate (w/v), 0.1% Triton X-100 (v/v)), incubated with MDW933 **5** (10 μL, 20 μM stock in DMSO) for 1h at 37°C and precipitated with chloroform/methanol (C/M, (9)). The protein pellet was rehydrated in 90 μL 8 M urea/100 mM NH_4HCO_3, reduced with 5 μL 90 mM dithiothreitol for 30 min at 37°C, alkylated with 7.5 μL 200 mM iodoacetamide for 30 min at RT in

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the dark and desalted by C/M. The pellet was dispersed in 100 µL 8 M urea/50 mM Na_2CO_3 buffer (pH 9.4). To 50 µL of the protein solution was added 5 µL hydroxylamine hydrochloride (10 M) and 45 µL 8 M Urea/50 mM Na_2CO_3 buffer (pH 9.2). The pH was adjusted to pH ~9.2 by the addition of NaOH (5M). The resulting mixture was incubated overnight at 37°C, precipitated with C/M, redissolved in 10 µL 8 M urea/100 mM NH_4HCO_3, diluted with 90 µL digest buffer (100 mM Tris-HCl pH 7.8, 100 mM NaCl, 1mM $CaCl_2$, 2% ACN) and digested with 500 ng trypsin overnight at 37°C. Peptides were collected and desalted on stage tips (10).

LC-MS analysis

Tryptic peptides were analyzed on a Surveyor nanoLC system (Thermo) hyphenated to a LTQ-Orbitrap mass spectrometer (Thermo). Gold and carbon coated emitters (OD/ID=360/25µm tip ID=5 µm), trap column (OD/ID=360/100 µm packed with 25 mm robust Poros®10R2/ 15 mm BioSphere C18 5 µm 120Å) and analytical columns (OD/ID=360/75 µm packed with 20 cm BioSphere C18 5 µm 120Å) were from Nanoseparations (Nieuwkoop, The Netherlands). The mobile phases (A: 0.1% FA/H_2O, B: 0.1%FA/ACN) were made with ULC/MS grade solvents (Biosolve). The emitter tip was coupled end-to-end with the analytical column via a 15 mm long TFE teflon tubing sleeve (OD/ID 0.3×1.58 mm, Supelco, USA) and installed in a stainless steel holder mounted in a nano-source base (Upchurch scientific, Idex, USA).

General mass spectrometric conditions were: an electrospray voltage of 1.8 kV was applied to the emitter, no sheath and auxiliary gas flow, ion transfer tube temperature 150°C, capillary voltage 41V, tube lens voltage 150V. Internal mass calibration was performed with air-borne protonated polydimethylcyclosiloxane (m/z = 445.12002) and the plasticizer protonated dioctyl phthalate ions (m/z = 391.28429) as lock mass (11).

10 µL of the samples was pressure loaded on the trap column with a 10 µL/min flow for 5 min followed by peptide separation with a gradient of 35 min 5-30% B, 15 min 30-60% B, 5 min A at a flow of 300 µL/min split to 250 nL/min by the LTQ divert valve. For each data dependent cycle, one full MS scan (300-2000 *m/z*) acquired at high mass resolution (60,000 at 400 *m/z*, AGC target $1x10^6$, maximum injection time 1,000 ms) in the Orbitrap was followed by 3 MS/MS fragmentations in the

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LTQ linear ion trap (AGC target $5x10^3$, max inj time 120 ms) from the three most abundant ions. MS^2 settings were: collision gas pressure 1.3 mT, normalized collision energy 35%, ion selection threshold of 500 counts, activation q = 0.25 and activation time of 30 ms. Fragmented precursor ions that were measured twice within 10 s were dynamically excluded for 60s and ions with $z<2$ or unassigned were not analyzed. Data from MS^2 was validated manually.

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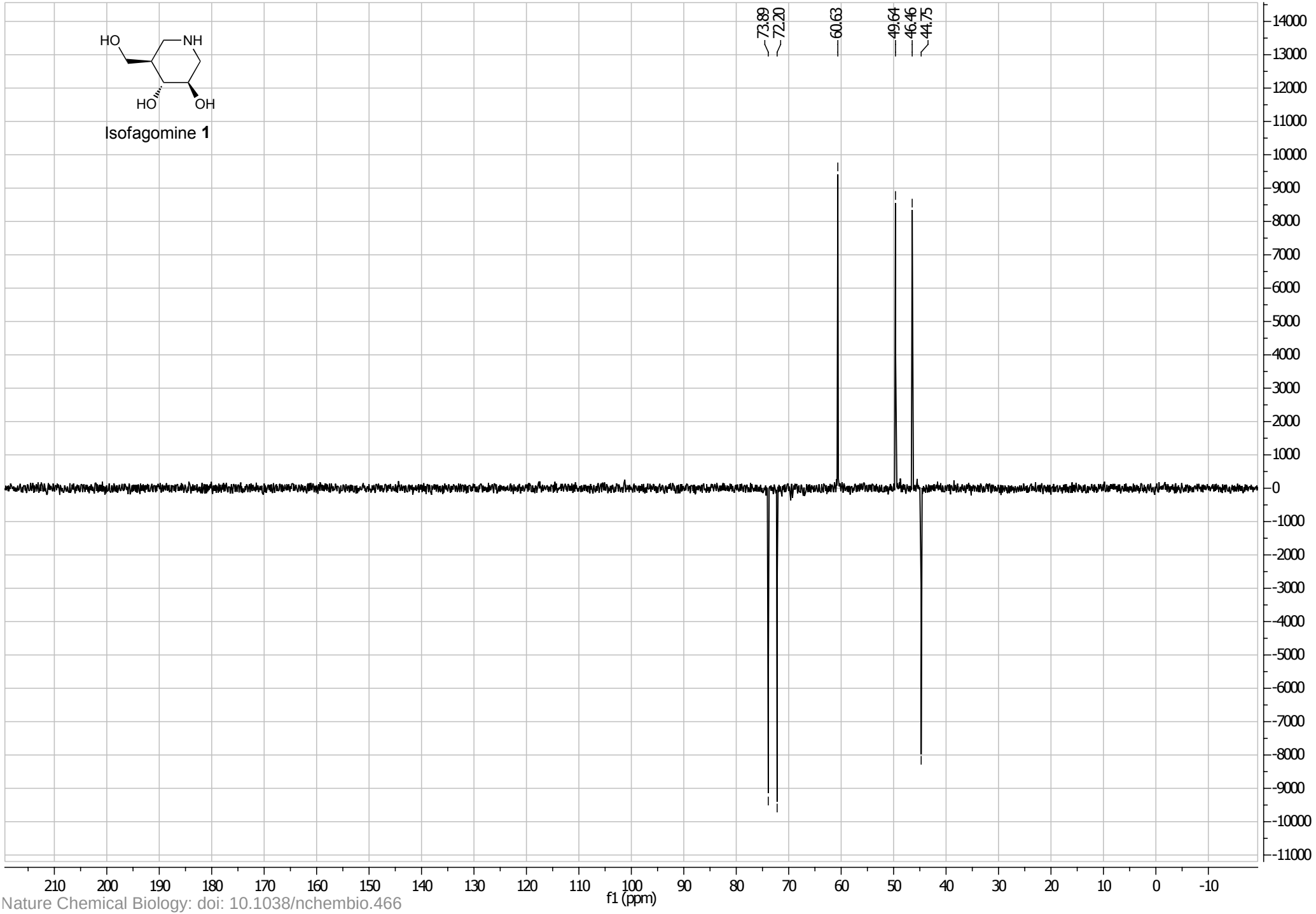

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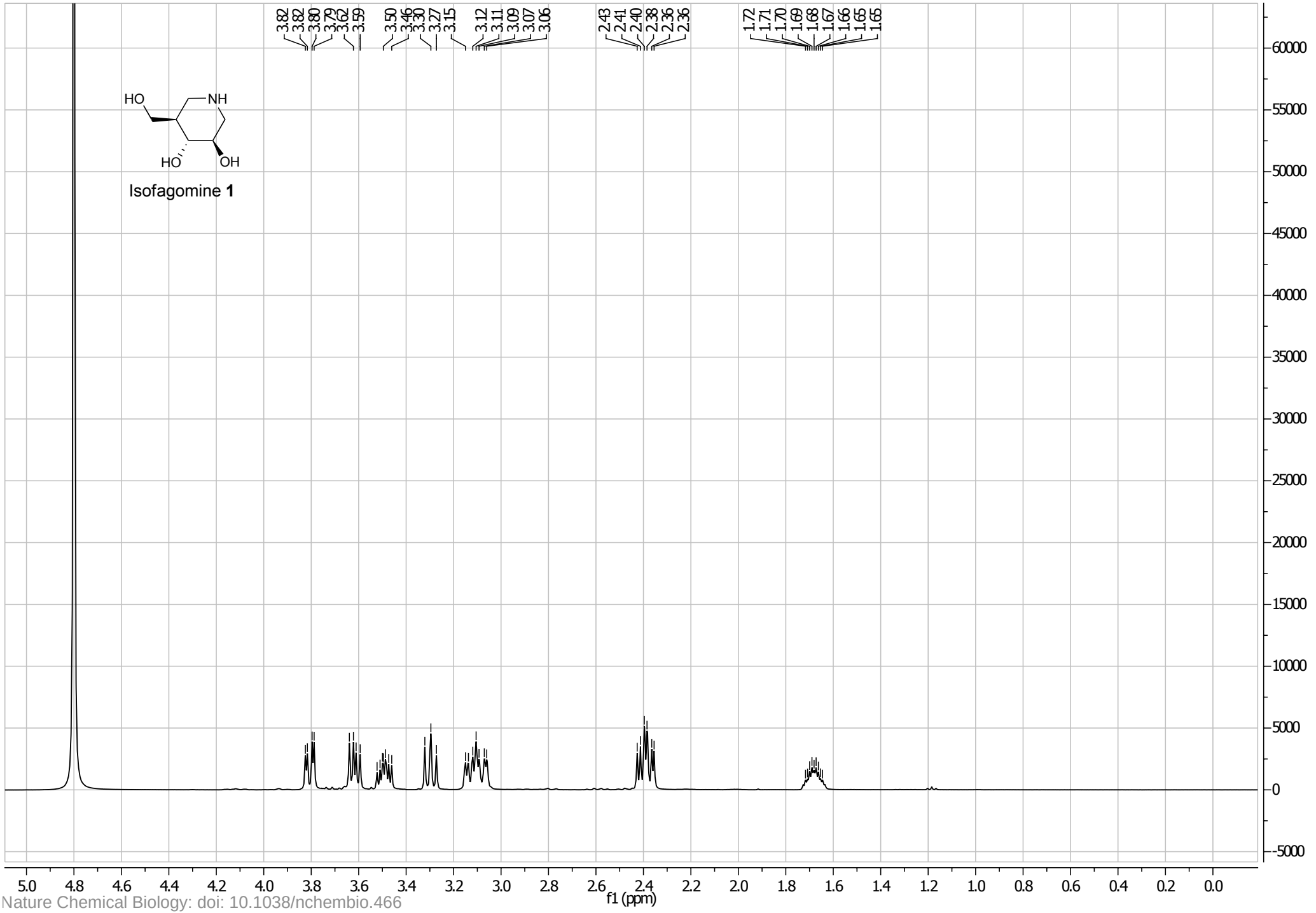

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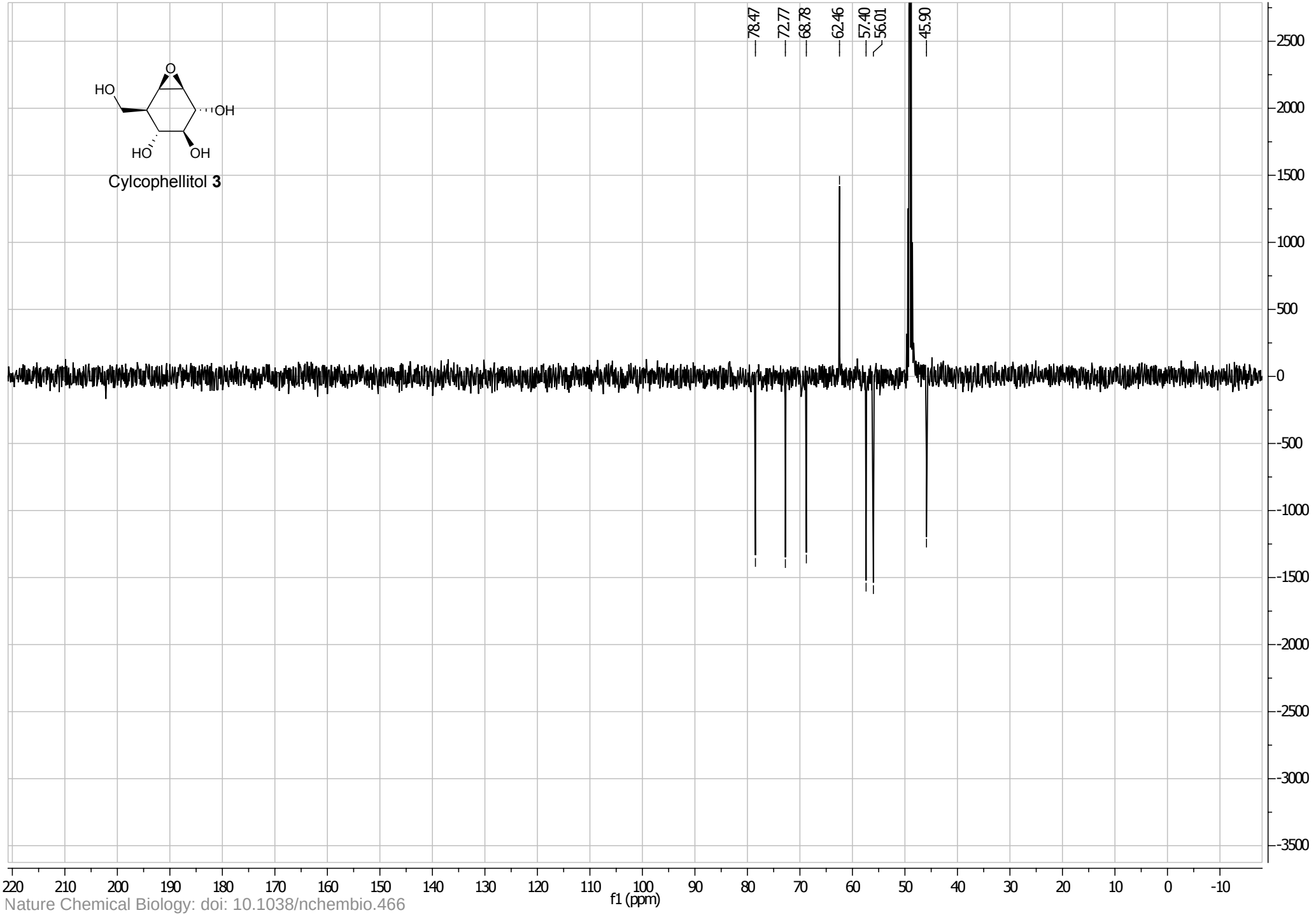

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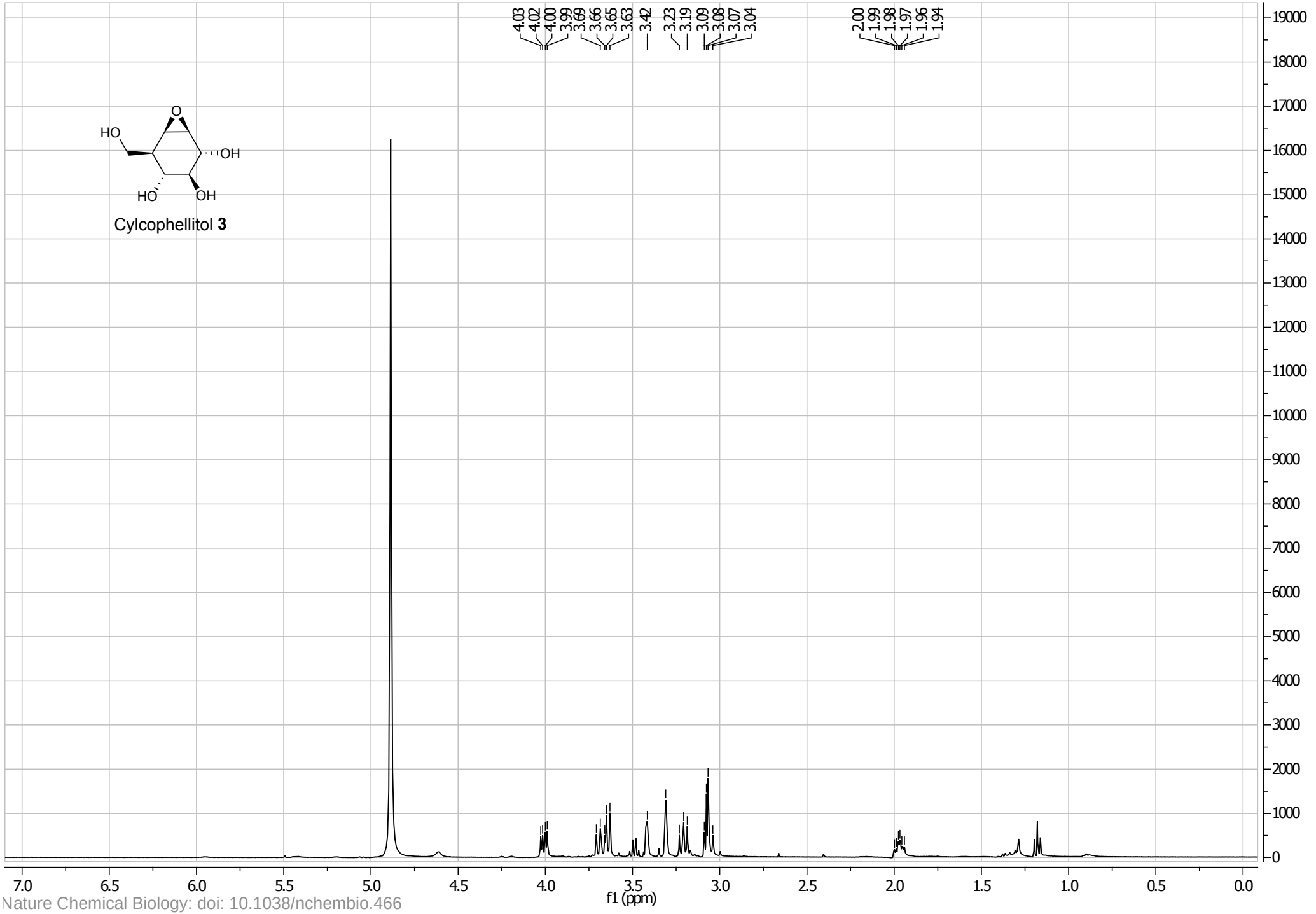

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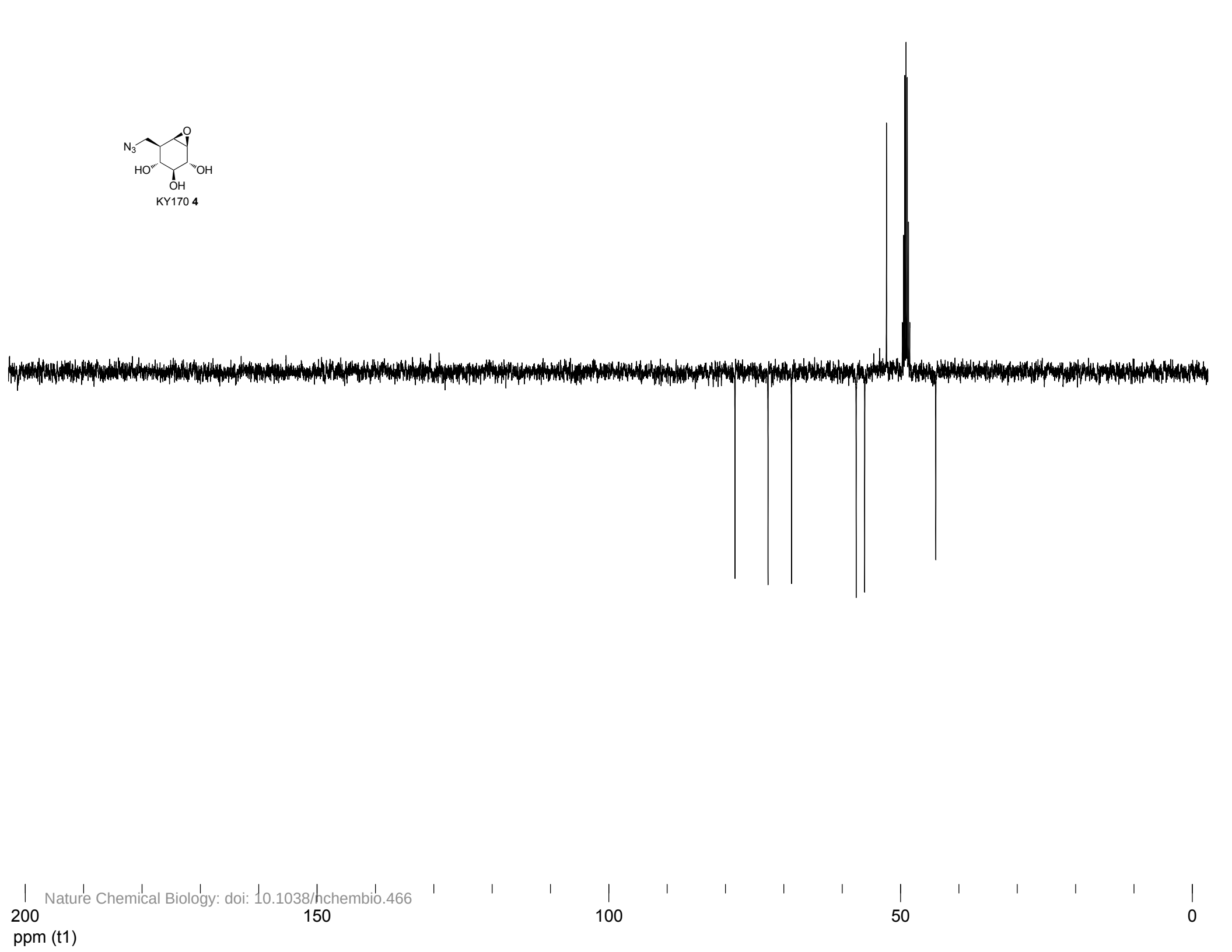
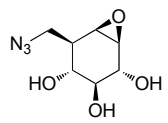
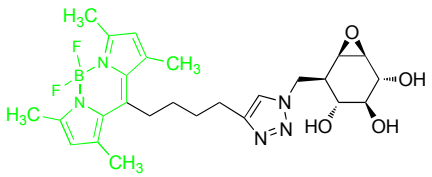




KY170 **4**

7.0 6.0 5.0 4.0 3.0 2.0 1.0 0.0

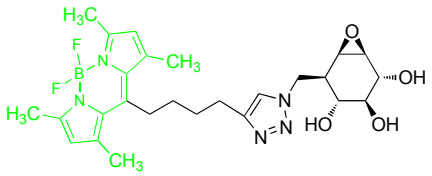
ppm (f1)



MDW933 **5**

200 150 100 50 0

ppm (f1)



MDW933 **5**

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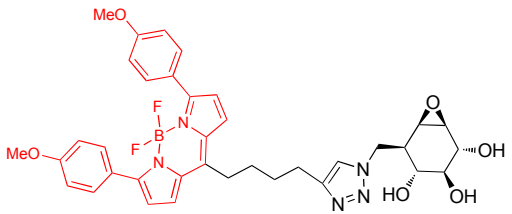
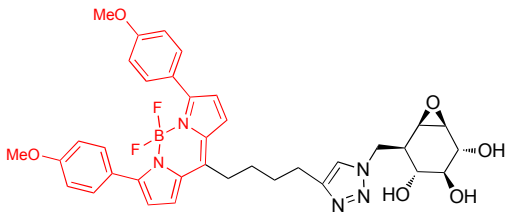
ppm (f1)



MDW941 **6**

150 100 50 0

ppm (f1)



MDW941 **6**

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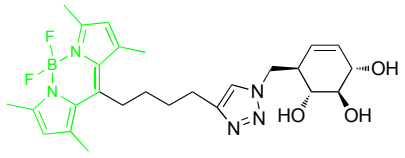
ppm (f1)



MDW1064 **8**

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ppm (f1)

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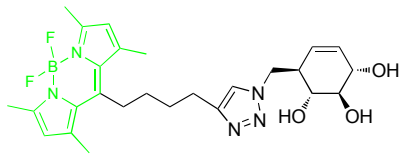
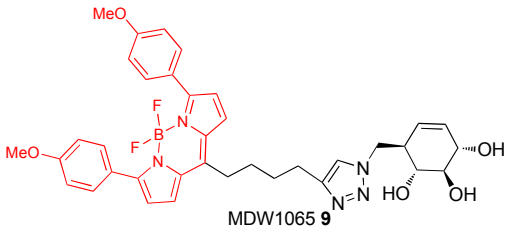


MDW1064

5.0

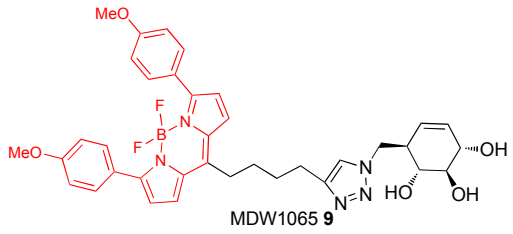
0.0

ppm (t1)



MDW1065 **9**

ppm (f1) 200 150 100 50 0



MDW1065 **9**

ppm (f1)

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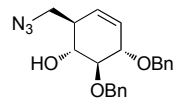
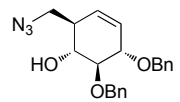


11

200 150 100 50 0

ppm (f1)



11

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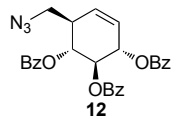
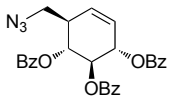
ppm (f1)



12

150 100 50 0

ppm (f1)



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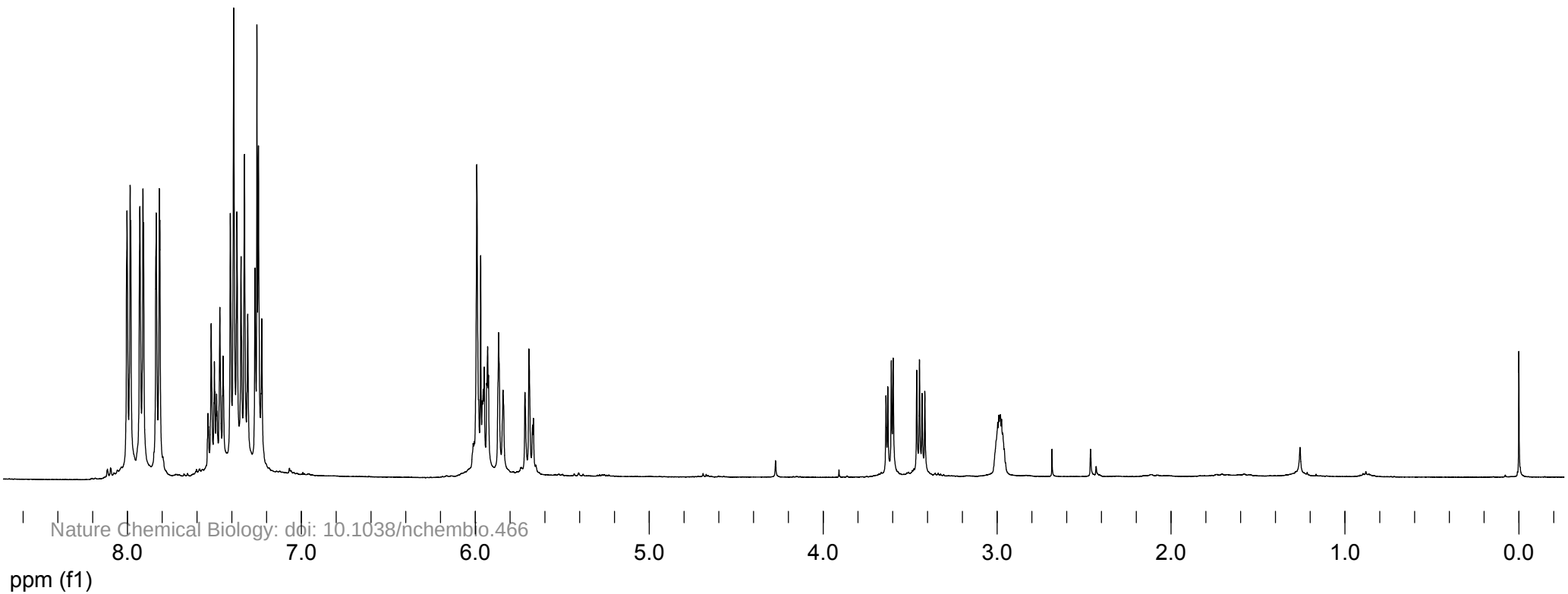

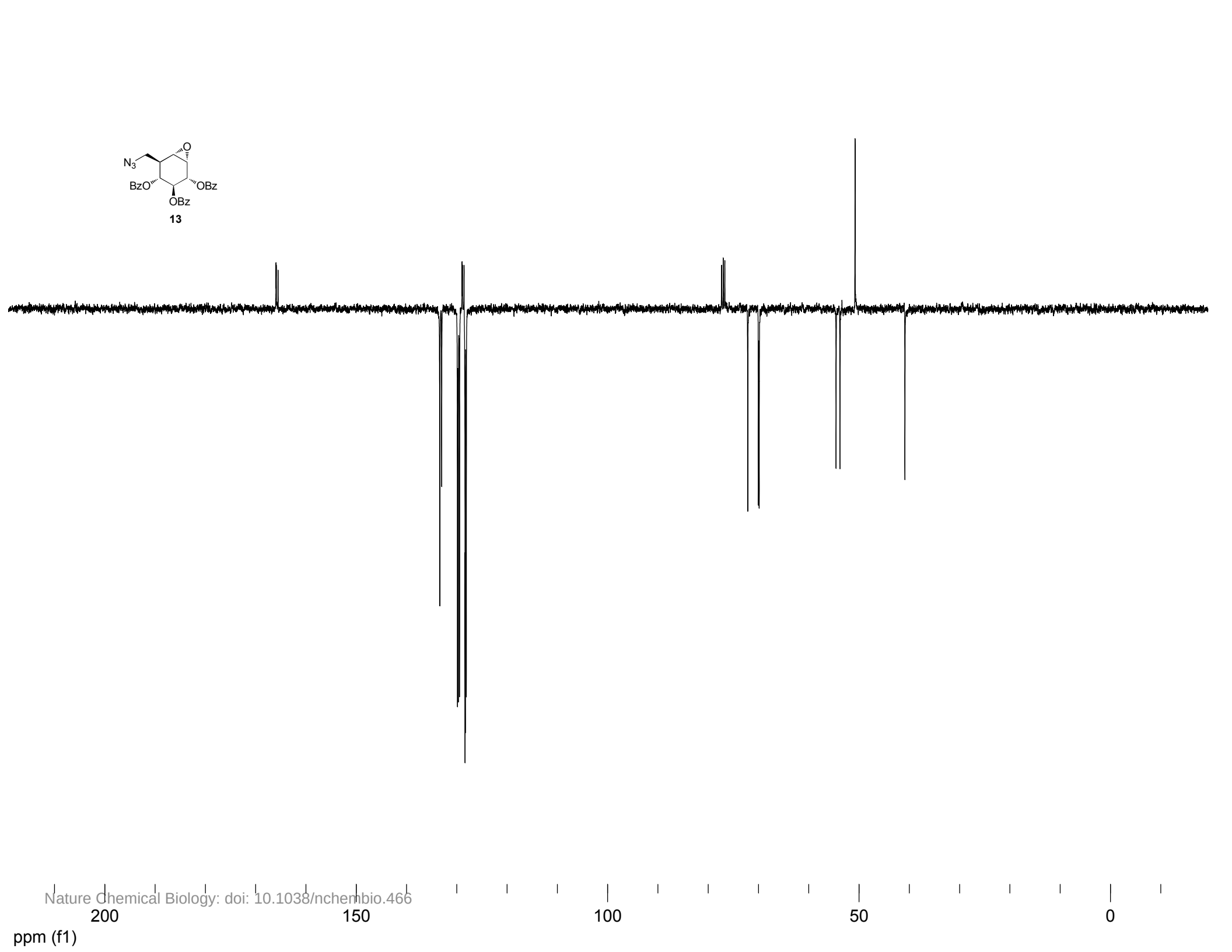

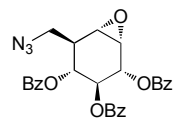

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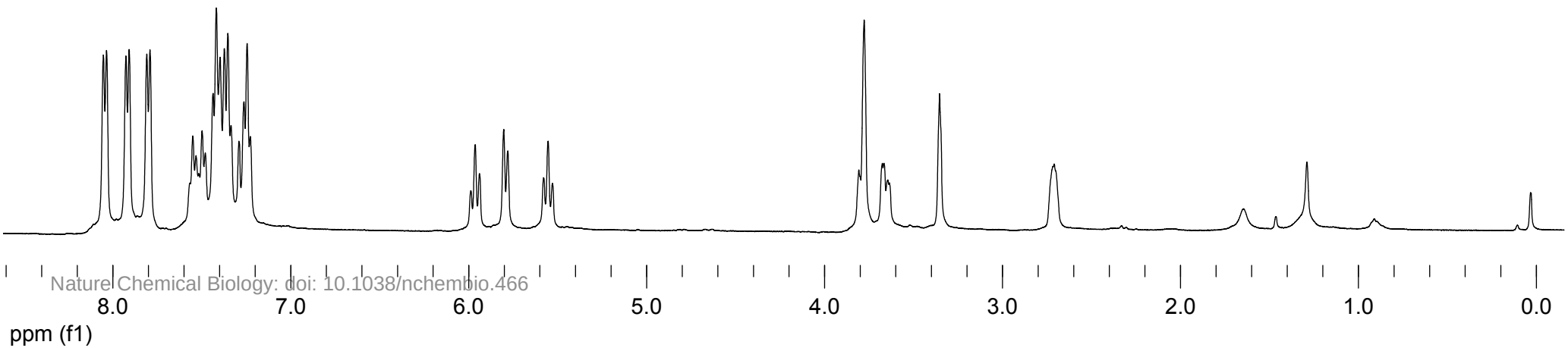
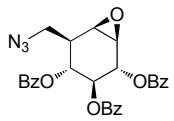
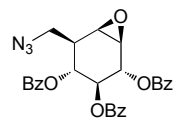




14

150 100 50 0

ppm (f1)



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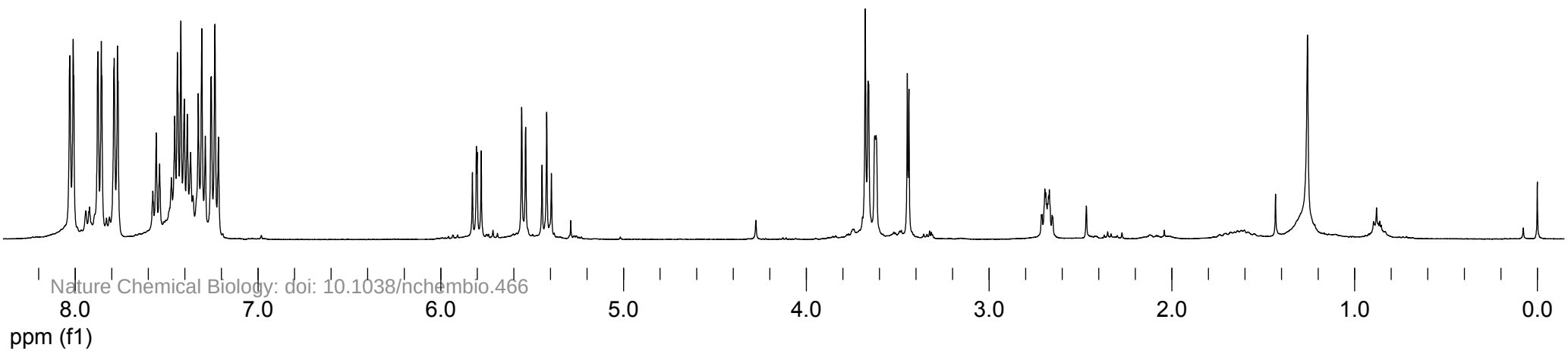

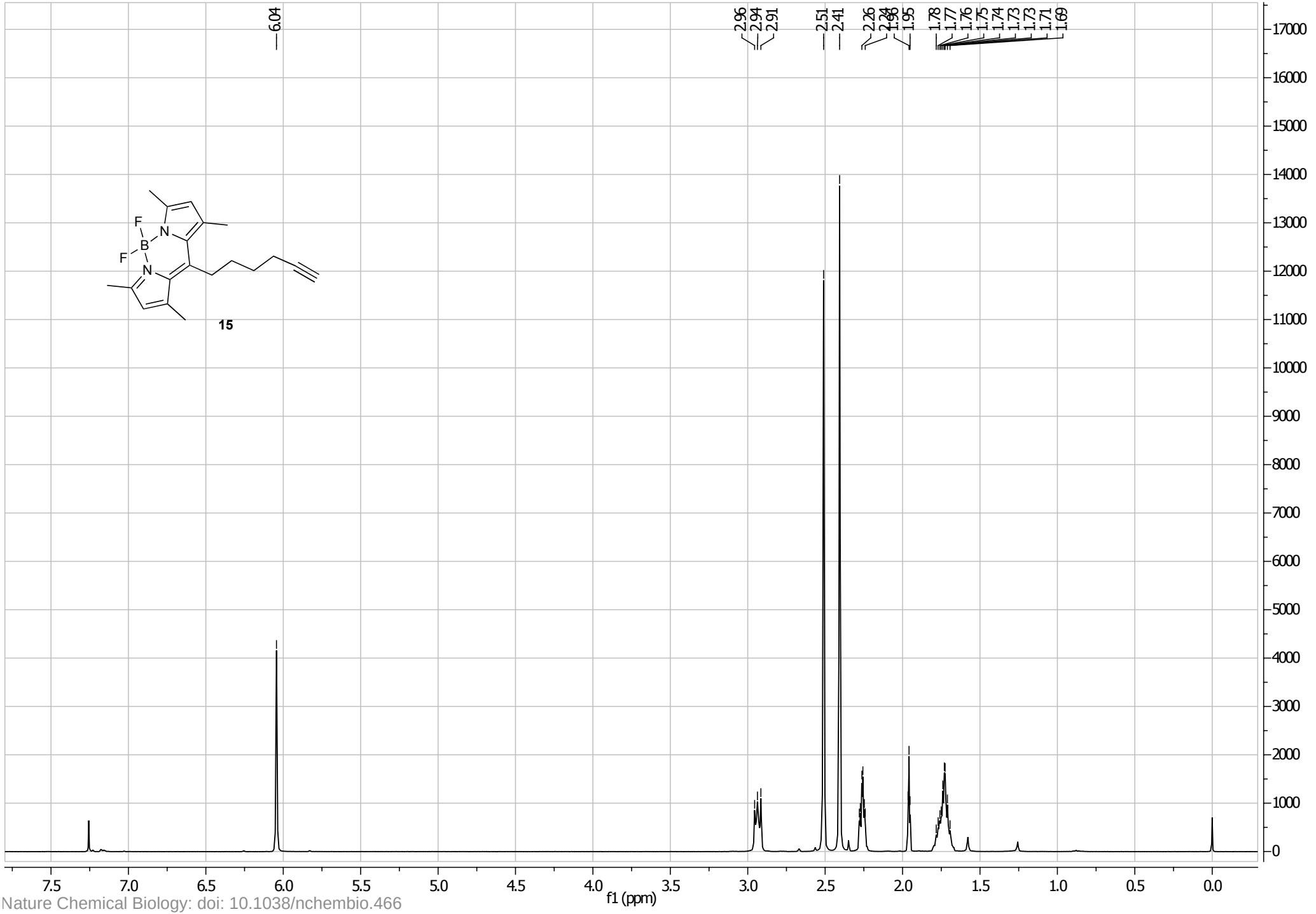

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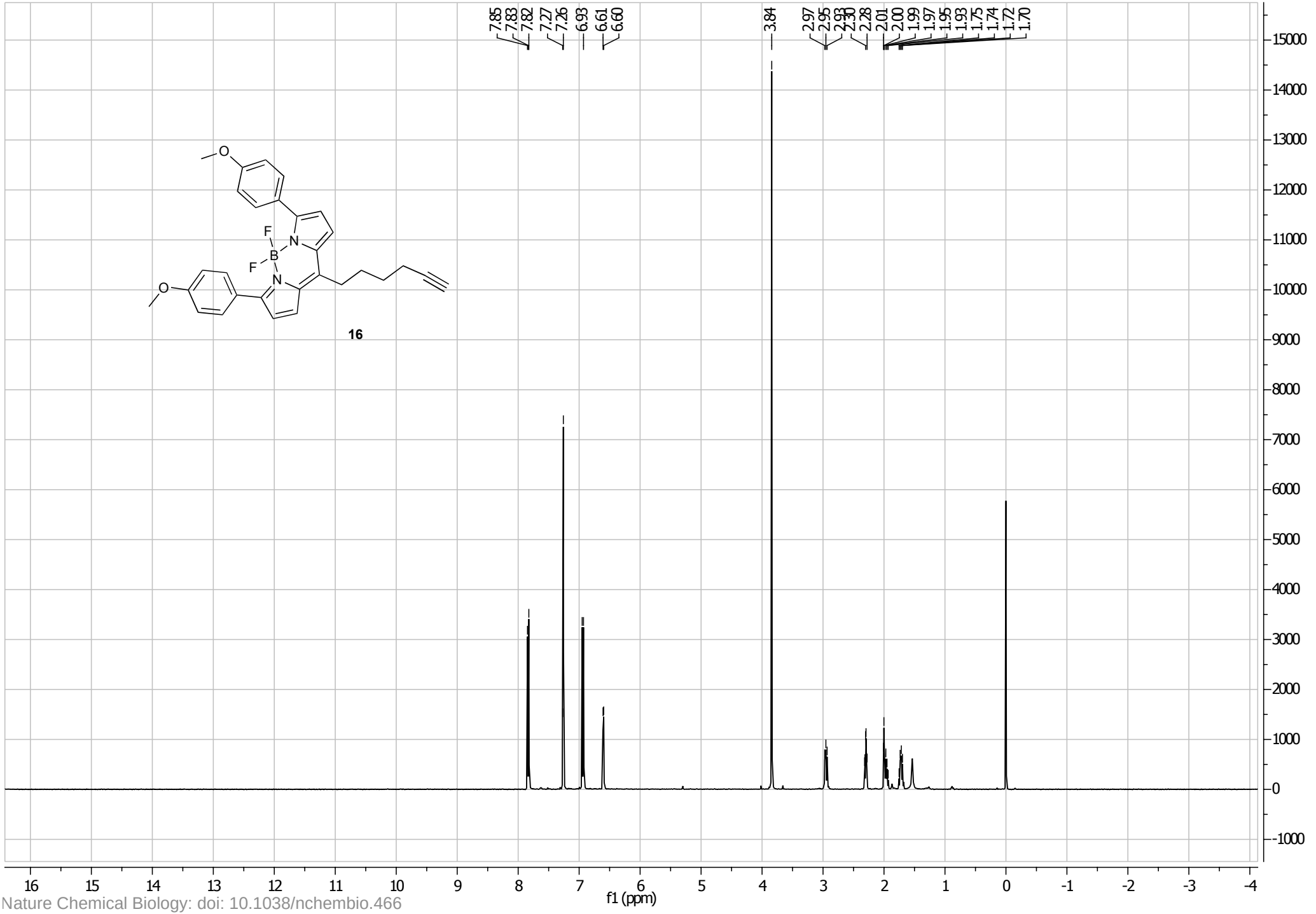

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